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THE HEART IN EXPERIMENTAL THYROTOXICOSIS*

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THERE are three entirely opposite schools of thought concerning the effects of thyroid disease on the heart. One group is of the opinion that thyrotoxicosis has no effect on the heart except to increase the metabolic activity.[1] Another group is inclined toward the view that the thyrotoxic disease causes myocardial damage, hypertrophy of the heart, and various clinical cardiac manifestations.[2] A third group, championed by more recent work, is of the opinion that the thyrotoxicosis acts only as a catalytic agent and brings to the surface latent cardiovascular lesions.[3]

Opinions vary not only as to the specificity of the possible thyrotoxic cardiac changes, but also as to the probable cause of such alterations in the heart. Some hold the primary cause as the toxicosis, while others attribute any cardiac changes to an indirect cause, such as the increased heart rate. The third group, as mentioned, attributes any possible changes to previous heart disease, especially a rheumatic myocarditis.

Thyrotoxicosis, experimentally induced, has not been entirely successful in solving the problem of the cardiac lesions in hyperthyroidism. Specific lesions have been produced in various animals.[4] However, others have reported that they were unable to demonstrate specific lesions in the hearts of experimental animals.[5]

Neither the clinical nor the experimental data at hand are sufficient to enlighten the problem of the effects of the thyroid upon the heart muscle in hyperthyroidism. In an experimental study somewhat similarly performed, Menne et al.,[5] found certain cardiac changes in the hearts of rabbits that were

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fed desiccated thyroid, but they concluded that it was possible that similar changes might be produced by cardiac overwork irrespective of the presence of thyroxin in the circulating blood. In an effort to investigate further this important and complex problem, the following experiments were undertaken.

METHODS OF INVESTIGATION

The work was performed entirely on rabbits. The animals were all of average weight and age. They were housed in a warm room and fed a normal rabbit diet, sufficient in quantity to ensure normal maintenance in weight which was determined by periodic weighings every four or five days.

Electrocardiographic tracings with Lead II were taken to determine accurately the pulse rate. Needle contacts were not employed, but a method with Sanborn Redux Paste was used. The hair on the areas of the right foreleg and left hindleg needed for contact were shaved and the paste rubbed thereon. Eight strand copper wire electrodes were employed, and these were wound twice around the selected area on the limb and again covered with the paste. Being painless, the method did not excite the animals, and tracings were obtained rapidly.

A normal electrocardiogram was obtained from each animal after it had become accustomed to being tied down on the animal board. Tracings were taken about every seven days, or thereafter, or until the death of the animal.

They were divided into six experimental groups, as follows: Group I, rabbits in which only the cardiac inhibitory nerves were removed; Group II, rabbits in which the cardiac inhibitory nerves were removed and digifoline (Ciba) injected subcutaneously; Group III, rabbits in which the cardiac inhibition was removed and quinidine sulfate administered orally; Group IV, desiccated thyroid (Armour) fed rabbits; Group V, thyroid fed and digifoline injected rabbits; and Group VI, thyroid and quinidine fed rabbits.

In the first three groups, where the cardiac inhibitory nerves were removed, it was performed in the following manner. The animals were given ether anesthesia, and a midline incision in the neck exposed the carotid sheaths. The pressor nerve of the vagus was isolated on each side, and a 1 to 2 cm. section was removed. The carotid arteries were stripped of their coverings up as far as the bifurcation into the internal and external branches and then swabbed with absolute alcohol. The animals were allowed to recover in a warmed room for several days.

Animals of one litter were used in the last three groups to exclude, as well as possible, any previous cardiac changes. The animals were all housed and fed similarly, and, in addition, were fed desiccated thyroid to produce toxicosis. The amount of thyroid fed varied with each individual animal, some becoming toxic on 2-3 grains per day and others requiring as much as 8 to 16 grains per day. They were fed the thyroid until the symptoms of toxicosis were manifested by weakness, diarrhea, loss of weight, prostration, lethargy, and increased pulse. Some of the animals, those that survived, were then given a rest period, and when the pulse and weight returned to normal, a second toxicosis was induced. This was continued until the animal died, or a sixty-day observation period passed, when the animal was killed. Normal electrocardio-

TABLE I

GROUP I. EXPERIMENTAL DATA CONCERNING RABBITS WITH CARDIAC INHIBITION REMOVED

ANIMAL NO.	WEIGHT KG.	DAYS	PULSE	WEIGHT LOSS	PULSE CHANGE	DEATH	HEART WEIGHT	LEFT VENTRICLE						RIGHT VENTRICLE						ECG	COURSE
								LENGTH	ABOVE	BELOW	RING	DIAM.	CIRC.	LENGTH	ABOVE	BELOW	DIAM.	CIRC.		
3	2.65	60	210	0	+30	K	9	22	6	4	12	14	28	22	1.5	1	14	28	Normal	Negative
4	2.8	60	250	0	+15	K	10	25	8	6	14	18	37	25	3	2	18	37	Normal	Negative
6	2.15	60	230	0	+10	K	10	29	8	6	12	14	29	29	2	1.5	14	29	Normal	Negative
7	2.62	60	210	0	+20	K	9	29	6	4	11	14	27	29	2	1	14	26	Normal	Negative
12	2.6	1	240	0	0	D	8.2	21	6	5	12	15	27	23	2.5	1.5	15	27	Normal	Carotid punctured
8	2.3	1	210	0	0	D	8	23	4	3	12	13	25	23	1.5	1.0	13	26	Normal	Vagus cut
9	3.0	3	240	0	0	D	12	27	6	3	14	16	29	27	1.5	1.0	16	30	Normal	Inf. Laryngeal cut
11	2.9	1	210	0	0	D	10	25	6	5	12	15	30	25	3	2	15	28	Normal	Vagus cut
16	2.4	7	210	0	+70	D	9	22	6	4	12	18	30	23	3.5	2	18	32	T waves negative	Suppurative pericarditis
18	2.48	0	170	0	0	D	8.7	20	6	5	11	16	29	20	3	1.5	16	29	Normal	Death due to anesthesia
23	2.92	0	160	0	0	D	9	23	6	4	13	20	36	23	2	1	20	25	Normal	Laryngeal obstruction

Heart weight is in grams.
All measurements are in millimeters.

graphic tracings were obtained as described, and tracings were taken every seven days thereafter.

Group I. Cardiac Inhibition Removed.—This group consisted of 11 rabbits that were operated in the manner described. Seven of the 11 animals died in one to seven days due to pulmonary complications, apparently resulting from the severing of other vagal branches besides the pressor nerve. Four animals survived and were killed at the end of the observation period. All were autopsied to exclude other conditions which might cause any cardiac pathology (Table I).

Although there were minor variations in certain cases, gross examination of the hearts in this group revealed that the muscle, in general, was moderately firm and the auricular chambers dilated. Epicardial fat was scant, and the ventricles appeared hypertrophic. The hearts of the seven animals found dead had dilated cavities filled with blood clots. The average weight of the hearts of the animals killed at the end of sixty days was 9.5 grams, and of those found dead, 9.4 grams. There was a difference in the thickness of the left ventricular musculature. In the animals killed it measured 7 mm. above and 5 mm. below, while in the animals found dead it was 5.7 mm. above and 4.3 mm. below. The length of the ventricles from the opening of the coronary artery to the tip of the apex was 3.1 cm. in the killed and 2.7 cm. in the animals found dead. The ventricular diameter was the same in both sets of rabbits, 1.6 cm.

For the microscopic examination three different sections were taken from all the hearts. One section was through the left ventricle, a second through the right ventricle, and the third section through a papillary muscle. In the animals that died shortly after operation no microscopic changes were noted in the heart muscle. In Rabbit No. 16, where the autopsy revealed a stitch abscess with penetration of the infection into the mediastinum and a resultant fibropurulent pericarditis, the microscopic examination of the heart showed hyalinization and separation of the muscle bundles with round and neutrophilic cellular exudation. In the four animals that survived the operation and were observed for sixty days, the findings were uniformly positive. These hearts showed hyalinization and granular degeneration with slight to moderate fragmentation and separation of the muscle bundles. Lymphocytic infiltration was noted in No. 3 and vacuole formation in No. 4 only.

Group II. Nerve Section and Digitalis.—The animals were operated on in the manner described and the pulse increase determined by the electrocardiogram. Ten days postoperative digifoline (Ciba, 2 c.c. equal 1 cat unit) injections were begun and each animal received two minims per pound of body weight daily. This was gradually increased until they received four to six minims per pound of body weight. Rabbit No. 14 had a pulse increase of 20 with the digitalis and died of over digitalization (Table II).

At the end of the sixty-day observation period, the three surviving animals were killed. Gross examination of the hearts revealed that the muscle was moderately firm, except in No. 14. Epicardial fat was scant or absent. The right heart chambers were dilated and the hearts appeared elongated. The heart weight averaged 7.7 grams. The ventricular length was 2.7 cm., the thickness of the left ventricle 6.5 mm. above and 5 mm. below, with a diameter of 1.6 cm.

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TABLE II

GROUP II. EXPERIMENTAL DATA CONCERNING RABBITS WITH CARDIAC INHIBITION REMOVED AND DIGITALIS INJECTED

ANIMAL NO.	WEIGHT KG.	DAYS	PULSE	WEIGHT LOSS	PULSE CHANGE	DEATH	HEART WEIGHT	LEFT VENTRICLE						RIGHT VENTRICLE					ECG	COURSE
								LENGTH	ABOVE	BELOW	RING	DIAM.	CIRC.	LENGTH	ABOVE	BELOW	DIAM.	CIRC.		
10	2.6	60	250	0	+20	K	6	19	7	5	10	12	23	19	1	0.5	12	32	P.O. Rate 270 A.D. Rate 150 Slurred QRS	Abscess at site of injection
12	2.6	60	200	0	+80	K	8	27	6	5	11	13	26	27	1	0.7	13	34	P.O. Rate 280 A.D. Rate 160 Slurred QRS	Negative
15	2.5	60	170	0	+60	K	7.8	25	8	5	13	20	35	25	2	1	20	30	P.O. Rate 230 A.D. Rate 150 Isoelectric T waves	Negative
14	2.86	28	190	0	+30	D	9	24	6	4	13	20	32	24	2	1	20	33	P.O. Rate 210 A.D. Rate 220 Depressed S-T intervals	Death due to overdigitalization

P.O. is the postoperative rate.
A.D. is the rate after digitalis.

The general findings on microscopic examination included poor staining of the nuclei, moderate separation of the muscle bundles with hyalinization, marked granular degeneration and fibrosis. In No. 10, where the animal developed a localized abscess in the left thigh, the findings were especially marked with vacuole formation, granular degeneration and separation of the muscle fibers.

Group III. Nerve Section and Quinidine.—The animals were operated in the manner described. Fourteen days postoperative feedings of quinidine sulfate were begun. The first doses were gr. ss, which was increased to gr. i after two weeks' medication (Table III). In the four rabbits autopsied at the end of the observation period, gross examination of the hearts revealed a firm muscle, purple-gray in color, and scant epicardial fat. The average heart weight was 5.7 gm. The length was 2 cm.; with thickness of the left ventricle 4.5 mm. above and 3.5 mm. below, and a diameter of 1.6 cm. Microscopic examination revealed essentially a moderate degree of granular degeneration and vacuolization, with a slight degree of fibrosis and hyalinization, separation of the muscle bundles, and fragmentation of a moderate degree.

Group IV. Thyroid Feedings.—Toxicosis was produced in these animals by feedings of desiccated thyroid. All of the animals showed emaciation and cachexia. On gross examination (Table IV) the heart muscles were moderately firm and devoid of epicardial fat. The right heart chambers were dilated and the hearts appeared elongated. Grayish areas were prominent over the external surfaces of the left ventricles. The average weight was 4.3 grams, length 2.1 cm., thickness of the left ventricle 4.8 mm. above and 3.3 mm. below, with a diameter of 1.3 cm.

In the animals that died early due to toxicosis there were no histologic changes prominent on the microscopic examination. There were focal areas of early degeneration, hyalinization, and slight separation of the muscle fibers. In the other animals the changes were more marked. These showed areas of marked granular and fatty degeneration with a foamy appearance, marked separation of the muscle fibers with fraying and fragmentation, cellular infiltration, both round cell and neutrophilic, hyalinization and fibrosis of these areas.

Group V. Thyroid and Digitalis.—These rabbits were given the digifoline subcutaneously for four days prior to the time the desiccated thyroid feedings were begun. The feedings were continued until the symptoms and signs of toxicosis became evident; the digitalis was continued each day also. None survived the toxicosis; all showed evidence of severe emaciation (Table V). Gross examination revealed elongated hearts with the muscles purple-gray in color and moderately firm. The right chambers were dilated and filled with clots. Epicardial fat was very scant or absent. The average heart weight was 5 grams. The length of the left ventricle was 2.2 cm.; thickness, 4.2 mm. above and 3.3 mm. below, with a diameter of 1.42 cm.

Where the animals died within twelve to sixteen days, following eight to twelve days of thyroid feedings, the histologic changes in the heart muscle were not marked on microscopic examination. There were focal areas of hyalinization and moderate granular degeneration with fragmentation and

TABLE III

GROUP III. EXPERIMENTAL DATA CONCERNING RABBITS WITH CARDIAC INHIBITION REMOVED AND QUINIDINE ORALLY

ANIMAL NO.	WEIGHT KG.	DAYS	PULSE	WEIGHT LOSS	PULSE CHANGE	DEATH	HEART WEIGHT	LEFT VENTRICLE					RIGHT VENTRICLE					QUINIDINE GIVEN	ECG	COURSE
								LENGTH	ABOVE	BELOW	RING	DIAM.	CIRC.	LENGTH	ABOVE	BELOW	DIAM.			
19	2.6	54	170	.15 kg.	+70	K	6.7	22	5	4	12	15	31	22	2	1	15	38 grains	Increased amplitude of T waves	Negative
21	2.6	54	160	.43 kg.	+80	K	7.0	20	5	4	13	19	33	20	1.5	1	19	38 grains	Increased amplitude of T waves	Negative
31	1.5	54	210	0	+30	K	4.8	20	4	3	12	16	30	20	2	1	16	88 grains	Increased amplitude of T waves	Negative
32	1.6	47	220	.4 kg.	+70	D	5.2	19	4	3	11	14	24	19	1	.5	14	72 grains	Increased amplitude of T waves	Negative

TABLE IV

GROUP IV. EXPERIMENTAL DATA CONCERNING RABBITS GIVEN DESICCATED THYROID

ANIMAL NO.	WEIGHT KG.	DAYS	PULSE	WEIGHT LOSS KG.	PULSE CHANGE	DEATH	LEFT VENTRICLE						RIGHT VENTRICLE						NO. OF TOXICOSIS	THYROID GIVEN	ECG	COURSE
							HEART WEIGHT	LENGTH	ABOVE	BELOW	RING	DIAM.	CIRC.	LENGTH	ABOVE	BELOW	DIAM.					
25	1.5	60	170	.5	+70	D	4.6	21	5	4	10	11	22	21	2	1.5	11		2	22 grains	High T's	Toxicosis
26	1.2	3	160	.2	?	D	3.2	20	4	3	9	13	23	20	1	1.0	13		1	5 grains	High T's	Toxicosis
27	1.3	5	150	.37	?	D	3.2	19	5	3	9	12	21	19	1	1.0	12		1	6 grains	High T's	Toxicosis
36	1.4	12	210	.4	0	D	4.1	14	5	3	9	12	24	14	2	1.0	12		1	75 grains	Normal	Toxicosis
37	1.3	14	200	.4	+40	D	3.7	20	5	3	10	11	21	20	1	1.0	11		1	93 grains	High T's and QRS	Toxicosis Toxicosis
38	1.4	50	250	.5	+110	D	5.3	23	5	3	12	15	28	23	1.5	1.0	12		2	118 grains	High T's and QRS	Toxicosis Toxicosis
39	1.7	56	210	.3	+180	K	5.8	23	4	3	13	14	32	23	1.5	1.0	14		2	138 grains	High T's	Toxicosis
41	1.2	13	210	.4	+20	D	3.8	18	4	3	11	13	23	18	2	1.0	13		1	87 grains	High T's	Toxicosis
40	1.3	42	240	.35	+90	D	4.4	20	5	4	10	12	20	20	2	1.5	10		2	274 grains	High T's	Toxicosis
35	1.6	49	250	.4	+100	D	5.0	23	6	4	12	15	32	23	2	1.0	15		2	300 grains	High T's	Toxicosis

TABLE V

GROUP V. EXPERIMENTAL DATA CONCERNING RABBITS FED DESICCATED AND DIGITALIS INJECTED

ANIMAL NO.	WEIGHT KG.	DAYS	PULSE	PULSE WITH DIGITALIS	PULSE WITH THYROID	DEATH	HEART WEIGHT	LEFT VENTRICLE						RIGHT VENTRICLE						DIGITALIS C.C.	THYROID GRAINS	ECG	COURSE
								LENGTH	ABOVE	BELOW	RING	DIAM.	CIRC.	LENGTH	ABOVE	BELOW	DIAM.						
46	1.7	18	220	220	310	D	4.8	22	5	4	12	14	29	22	2	1	14	36	126	T waves + to −	Toxicosis
47	1.2	12	200	240	320	D	4.3	21	4	3	11	13	26	21	2	1	13	28	83	T waves negative	Toxicosis
48	1.1	16	210	210	280	D	4.1	20	3	2	10	15	29	20	1.5	1	15	32	72	P-R prolonged	Toxicosis
49	1.3	16	270	230	340	D	6.0	21	4	3	12	16	30	21	2	1	16	32	72	T isoelectric	Toxicosis
50	1.6	15	220	240	310	D	5.6	20	5	4	11	13	26	20	2	1	13	30	147	Low S-T	Toxicosis

TABLE VI

GROUP VI. EXPERIMENTAL DATA CONCERNING RABBITS FED DESICCATED THYROID AND QUINIDINE

ANIMAL NO.	WEIGHT KG.	DAYS	PULSE	PULSE WITH QUINIDINE	PULSE WITH THYROID	DEATH	HEART WEIGHT	LEFT VENTRICLE						RIGHT VENTRICLE						QUINIDINE GRAINS	THYROID GRAINS	ECG	COURSE
								LENGTH	ABOVE	BELOW	RING	DIAM.	CIRC.	LENGTH	ABOVE	BELOW	DIAM.						
51	1.4	10	220	210	310	D	4.7	23	3	2	11	13	25	23	1.5	1	13	20	58	No change	Toxicosis
52	1.7	15	200	200	270	D	5.5	22	4	2	12	13	25	22	2.0	1	13	30	105	No change	Toxicosis
53	1.2	10	170	140	250	D	6.3	22	3	2	13	17	28	22	1.5	1	17	20	55	No change	Toxicosis
54	1.7	10	180	210	290	D	6.5	22	4	3	10	15	26	22	1.0	.75	15	20	70	High T's	Toxicosis
55	1.6	20	180	170	320	D	6.1	22	6	4	10	15	25	22	1.5	1	15	40	170	High Voltage	Toxicosis

fraying of the muscle. Round and neutrophilic cellular infiltration were noted in two of the animals. The rabbit, No. 46, that lived the longest, 18 days, showed the most marked degenerative changes, which were fatty and graular, with vacuolization, moderate fibrosis, and marked neutrophilic and endothelial cellular exudation.

Group VI. Thyroid and Quinidine.—These animals were first fed quinidine for four days before desiccated thyroid was given. Both were then fed until the symptoms and signs of toxicosis were manifested. All of the animals died during the first toxicosis (Table VI). Gross examination revealed elongated hearts with dilated right chambers and scant epicardial fat. The muscles were purple-red and firm. The average heart weight was 5.8 grams. Average length was 2.2 cm.; thickness, 5 mm. above and 2.1 mm. below; and the average ventricular diameter was 1.5 cm. Many marked histologic changes were noted on microscopic examination. These consisted of moderate to marked fraying and fragmentation of the muscles with hyaline and granular degeneration, and cellular infiltration of the round and neutrophilic types.

ANALYSIS OF EXPERIMENTAL DATA

Several interesting points were brought out in this experimental study. In Group I, where the operation for the removal of cardiac inhibitory nerves was performed on 11 rabbits, only four survived the observation period of sixty days. Here there was only the increased heart rate with increased work for the myocardium. Apparently the tachycardia alone caused an increase in the ventricular measurements of the animals that lived sixty days. In Group II, animals that had the nerve section performed and received digitalis ten days later, the picture was practically similar. The average heart weight was not as high as in the first group, the cardiac rate having decreased after the tenth postoperative day due to the digitalis. In Group III, where the nerves were removed and quinidine fed, no great deviation was noted in the anatomical findings.

Cardiac changes became more manifest in the last three groups that were fed desiccated thyroid. The average weight loss was about the same. There was no doubt as to the evidence of an increased metabolism accompanied by an increased respiratory rate and an accelerated heart action. The longer the animals lived with the toxicosis, the greater were the cardiac changes. The hearts appeared elongated, and the histologic changes were considerably greater than in those animals who received no thyroid feedings.

COMMENT

There are many factors to be considered in the effects of thyrotoxicosis on the heart. It is generally agreed that thyrotoxicosis produces general muscular weakness. It uses up glycogen reserves so that endurance is markedly lessened from the overactivity. The heart usually shares this decrease in glycogen reserve and suffers according to the extent and duration of the loss. Tachycardia is due mainly to the increase of thyroxin in the myocardium which causes the heart to beat more rapidly and more vigorously.[6] However, tachycardia alone is not sufficient to cause marked cardiac alteration. It may produce

some changes, such as fragmentation and degeneration of the muscle, depending on its duration. It is a factor, secondary to thyroxin.

There was a great variety and a marked degree of alterations in the heart muscle. Whether the compensating and neutralizing capacity of the neuro-endocrine system accounts for some of the variations can only be theorized at this time. The quantitative results were too variable for a direct ratio correlation. Even in the experimental animal, there is a different degree of tolerance to thyroid medication, and it is plausible that this is caused by a synergistic and antagonistic interrelation of other endocrine glands.

Experimentally, in rabbits, thyroid extract produces definite myocardial changes in varying amounts. These changes are not proportional to the alteration of the heart rate alone nor dependent on a previous demonstrable infection. Nor is the myocardial damage proportional to the increased rate of speed per second.[5] Even though the cardiac lesion in thyrotoxicosis may not be specific or constant, the myocardial damage encountered, however the mechanism, is primarily due to the effects of increased thyroid on the heart muscle itself.

SUMMARY

Experimentally induced thyrotoxicosis in rabbits resulted in the following changes in the heart: parenchymatous and fatty degeneration, cellular invasion, fraying and fragmentation of the heart muscle, and fibrosis. Similar changes, but not as marked, were produced by cardiac overwork without an excess of thyroxin in the circulation. This was produced by cutting the depressor nerves and denuding the carotid sinuses of their investments in order to allow the heart to operate uncontrolled.

A heart that is induced to work more rapidly does respond with morbid anatomical changes, but these cardiac alterations are not as marked as those due to the summation of the effect of increased thyroxin on the myocardium and the tachycardia, which is again a thyroxin effect.

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FATAL ANURIA FOLLOWING ADMINISTRATION OF SULFONAMIDES WITH REFERENCE TO TUBULAR NECROSIS AND REGENERATION*

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IN MOST reports on the effects of the sulfonamide drugs on the urinary tract, emphasis has been given to the deposition of crystals in the tubules of the kidney and the formation of concrements in the pelves and ureters. Less emphasis has been given to the direct toxic effect of these drugs on the renal epithelium. In experiments on the toxicity of sulfathiazole in mice, Rake, van Dyke, and Corwin[1] found only an occasional mouse in which there were advanced degenerative changes and necrosis in the epithelium of the proximal convoluted tubules. Similar observations are reported by Kolmer[2] in studies with sulfathiazole in rabbits. Tubular necrosis was found only in animals receiving the largest doses—0.2 Gm. per kilogram twice a day for ten days. Hellwig and Reed[3] emphasize the toxic effect of sulfadiazine on the convoluted tubules and distinguish between this effect and mechanical blocking of the tubules by crystals. The latter effect is stressed by Bradford and Shaffer,[4] who accent the need for early ureteral catheterization and pelvic lavage. Hellwig and Reed insist that lavage in their case was of no avail—that the lesion was one of toxicity instead of mechanical effect.

In a recent case studied at the St. Louis Children's Hospital the preponderance of clinical and pathologic evidence indicates that death from renal insufficiency was directly related to degenerative changes in the renal epithelium, and that the deposition of crystals in the kidney and pelvis was of minor importance.

CASE REPORT

G. S., a boy of nine months, was seen first in the Out-Patient Department on March 10, 1942, with acute rhinopharyngitis, bilateral catarrhal otitis media, and bronchitis. His temperature was 39° C. Sulfathiazole 0.25 Gm. every four hours (0.2 Gm. per kilogram), neo-

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synephrin nose drops, and an adequate fluid intake were prescribed. The temperature returned to normal within twenty-four hours, the infection cleared rapidly, and the sulfathiazole was discontinued on the fifth day. On this same day gross hematuria and sulfathiazole crystals in the urine were noticed, both of which disappeared within the next twenty-four hours. Three days later the temperature rose again to 39° C. because of recurrence of the otitis media. This time treatment was by bilateral myringotomy and sulfadiazine, 0.25 Gm. every four hours. The temperature again returned to normal within twenty-four hours, and the otitis media began to subside. No hematuria was noticed. On the fourth day of the relapse, vomiting occurred, and when the patient was hospitalized, examination revealed marked oliguria, considerable albuminuria, and 4 to 5 red blood cells per high-power field in a centrifuged specimen but no crystals of sulfadiazine. During the second twenty-four hours of hospitalization the temperature rose, only a few cubic centimeters of urine were voided, and generalized edema developed. The heart sounds were normal. Late the second day the kidneys were found to be enlarged and tender. On the third day the heart tones became poor, cyanosis and marked restlessness developed, and the child died fifty-four hours after hospital entry, despite administration of magnesium sulfate, oxygen, and sedation. Additional laboratory work included a negative Kline test; hemoglobin 11.3 Gm.; red blood cell count 4.5 million; white blood cell count 13,000; and Schilling differential of 6 per cent stabs, 55 per cent segmented, 37 per cent lymphocytes, and 2 per cent monocytes.

TABLE I

DAY	CHEMOTHERAPY	URINE	DRUG LEVEL (MG./100 C.C.)	
			BLOOD	URINE
March 10	Sulfathiazole 0.25 Gm./4 hr.			
March 11, 12, 13	Same			
March 14	Same	Gross hematuria Numerous crystals		
March 15	None	Cleared gradually		
March 16	None	Clear*		
March 17	None	Clear*		
March 18	Sulfadiazine 0.25 Gm./4 hr.	Clear*		
March 19, 20	Same	Clear* Amount (?)*		
		PATIENT HOSPITALIZED		
March 21	Same	Oliguria noted	Free 17.7 Total 21.2	
March 22	None	Marked oliguria Albumin, +2 R.B.C. centrifuged Crystals—none		
March 23	None	Marked oliguria	A.M.— Free 15.8 Total 29.4 P.M.— Free 16.5 Total 20.5	Free 168.0 Total 375.0

*As observed by the mother.

The autopsy (Washington University No. 9746) was performed two and one-half hours post mortem. The body was edematous; the face, especially about the eyes, was puffy due to subcutaneous edema. The serous cavities contained excessive amounts of clear yellow fluid without evidence of inflammatory lesions within them. The kidneys were similar; the right weighed 55 Gm. and the left 53 Gm. The capsules were tense, and during the process of section of the organs large amounts of reddish yellow fluid spurted around the cutting edge

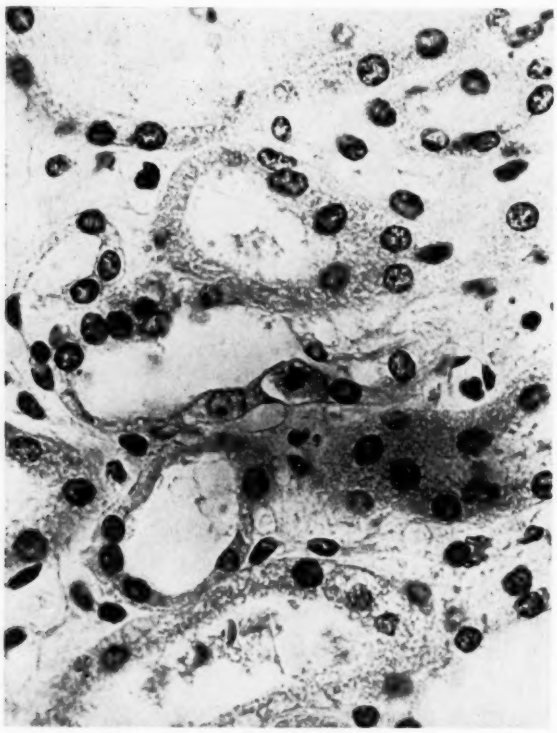

Fig. 1.—A mitotic figure is present in an epithelial cell in the center. In other tubules the cell membranes are indistinct and nuclei have disappeared.

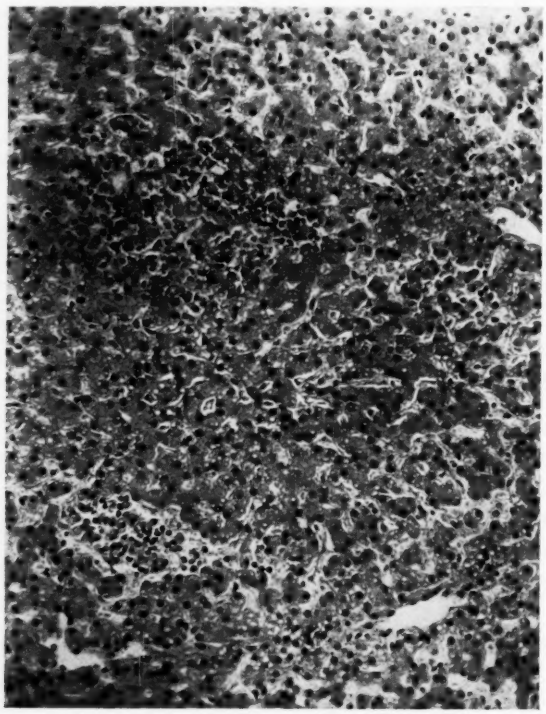

Fig. 2.—Focal necrosis in the liver. The hepatic cells are destroyed in these areas and there is an infiltration of leucocytes, lymphocytes, and a few monocytes.

of the knife. Approximately 10 c.c. of fluid were in the pelves and calices of these kidneys. The architecture of the organs was not unusual. There was a slight amount of dilatation of the calices and pelves. In the grayish white mucosa of the pelves were petechiae, and on the surface of the mucosa were many small yellow crystals. At the orifices of the ureteropelvic junctions were large aggregations of these crystals. These crystals were also found on the ureteral mucosa. The ureters were otherwise normal. The urinary bladder contained no urine. The remainer of the gross pathologic findings are listed below in the complete pathologic diagnosis.

Microscopic examination of the kidneys reveals moderate necrosis of the epithelium of the tubules, particularly of the proximal convoluted tubules. Within the lumen of the tubules are desquamated cells, eosinophilic debris, and occasionally red blood cells. Often there are syncytial masses of cytoplasm congregated at one end of a section of tubule with 4 to 8 well-preserved chromatic nuclei. Mitotic figures in the epithelial cells of the proximal tubules are moderately abundant (Fig. 1). Near the pelvic margins of the medullary papillae is noticeable dilatation of the tubules. Hemorrhage is present beneath the mucosa and within the adjacent tubules. The glomeruli are of moderate size, and the epithelial cells of the visceral layer of Bowman's capsule are cuboidal in shape, with deeply chromatic nuclei. Within Bowman's space there are a few desquamated necrotic cells and eosinophilic debris. There is slight interstitial edema of the cortex.

In the liver are many areas of focal necrosis (Fig. 2). These areas are small and contain necrotic liver cells, leucocytes, lymphocytes, and a few mononuclear cells. There is a moderate amount of cloudy swelling and fatty degeneration of the hepatic cells.

The complete pathologic diagnosis is as follows: Primary: Acute nephrosis with early regeneration of tubular epithelium; crystalline material in the pelves and ureters; hydronephrosis, bilateral; petechiae and ecchymoses of the mucosa of the pelves of the kidneys; edema of the subcutaneous tissues; ascites; hemorrhage of the middle lobe of the right lung; petechiae of the myocardium; hydrothorax, bilateral; focal atelectasis of the lungs; hyperplasia of the intrapulmonary lymphoid follicles; partially organized thrombi in the smaller branches of the pulmonary arteries. Accessory: Patent foramen ovale, guarded type, and immaturity of the kidneys.

COMMENT

The pathologic findings in the kidneys of this child indicate that there are two distinct lesions: one the result of crystallization of sulfathiazole in the ureters, pelves, and tubules; and the other, necrosis of the epithelium of the proximal convoluted tubules with early regeneration. It is not possible to attribute the anuria to either of these exclusively, but the preponderance of evidence indicates that the degenerative changes in the renal epithelium are more important. This conclusion is supported by the finding of regeneration, which might be expected to occur on the eighth to the tenth day following primary damage to the kidney, as in the case of poisoning with bichloride of mercury. In this patient the first sulfonamide was administered on March 10, and four days later there were gross hematuria and numerous crystals in the urine. Oliguria appeared on March 21, eleven days after the administration of sulfathiazole and three days after the first dose of sulfadiazine. It is improbable that the renal epithelium could have been damaged and could have showed beginning repair within the period of five days. It must then be concluded that the injury to the kidney resulted from the sulfathiazole given from March 10 to March 14, inclusive.

The sulfathiazole was given in therapeutic amount, and the reason for the damage to the kidney is not evident. It is possible that the mother did not give

the child an adequate amount of water, and that the concentration of the acetylated derivative in the urine was greater than in the usual case. There is also the possibility that the relative immaturity of the kidney may have played a role. The epithelium covering the visceral layer of Bowman's capsule was, in this nine-month-old child, still of the cuboidal type seen in newborn infants.

SUMMARY

1. A case of fatal anuria following the administration of therapeutic doses of sulfathiazole is reported.

2. An analysis of the pathologic findings indicates that injury to, and necrosis of, the renal epithelium were of greater physiologic importance than the usually emphasized formation of concrements and obstruction of the ureter and tubules.

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CIRCULATION TIME: A REVIEW OF PREVIOUS METHODS AND THE INTRODUCTION OF AMINOPHYLLIN AS A NEW AGENT

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TECHNICAL difficulties have thus far interfered with direct measurements of the velocity of blood in the human being. An approximation has been suggested by the estimation of the "circulation time" which obviously varies inversely with the velocity. The estimations of circulation time are dependent upon the detection of the arrival at some previously chosen point of a substance introduced elsewhere.

The arrival of the substance may be detected by the patient, by the observer, or both. In general, it is expected that objectivity represents not only greater accuracy, but also wider applicability (children, uncooperative or comatose patients).

The earliest effort to measure circulation time was that of Hering[1] in 1827. Using the Prussian blue method he measured the time lapse between the injection of potassium ferrocyanide in one jugular and its appearance in the other.

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In 1858 Vierordt[2] amplified and extended the use of this method and, after many experiments in different animals, predicted that in a human being with a heart rate of 72 per minute the circulation time from jugular to jugular would be 23.1 seconds.

In 1893 Stewart[3] injected methylene blue intravenously and detected its arrival elsewhere by transillumination. He also injected hypertonic sodium chloride solution, the presence of which was determined by the use of a galvanometer connected to nonpolarizable electrodes placed on a vessel. Meldolesi,[4] in 1925, and Koch,[5] in 1928, used the same method.

In 1922 Loevenhart[6] and his co-workers injected sodium cyanide intravenously and used a respiratory gasp as the end point at which time the cyanide was considered to have reached the carotid sinus. In 1933 Robb and Weiss[7] adapted this method for use in human beings. In normal subjects they found it to vary between 9 and 21 seconds, with an average of 15.6. The foot-to-carotid time averaged 27.7 seconds. This circulation time includes the time taken for the reaction to occur after the arrival of the cyanide at the carotid sinus. Olson, Levinson, Necheles, and Gutmann[8] in a study of circulation time in shock showed that the "reactivity time" of sodium cyanide varied constantly between 1 and 2 seconds.

In 1927 Blumgart and Yens[9, 10] devised a method for determining vein-to-artery and vein-to-heart time which made it possible to calculate the circulation time in the pulmonary circuit. They injected radium C into an antecubital vein and by means of a modified Wilson cloud chamber observed the time required to reach the heart and the opposite brachial artery, near the elbow. Blumgart and Weiss[11] found that the time from vein to artery varied from 12 to 24 seconds, with an average of 18 seconds. The pulmonary circuit time varied from 5 to 17 seconds, with an average of 10.8 seconds. This method undoubtedly is the most accurate one available, but its complexity makes its clinical use unfeasible.

Weiss, Robb, and Blumgart,[12] in 1929, found that the antecubital injection of histamine phosphate produced a flushing of the face upon the arrival of the histamine in the minute vessels. The arm-to-face time averaged 24 seconds. The method has been abandoned because of dyspnea, cardiac asthma, and headache as reaction phenomena.

Winternitz, Deutsch, and Brüll[13, 14] found that the injection of a solution of sodium dehydrocholate (decholin) produced a bitter taste upon arriving in the tongue capillaries. Tarr, Oppenheimer, and Sager[15] found the circulation time by this method to vary between 10 and 16 seconds.

In 1933 Fishberg, Hitzig, and King[16] used saccharin to measure the arm-to-tongue circulation time. A sweet taste signals the end point. The time by this method varies between 9 and 16 seconds.

In 1935 Teplov and Sor[17] used lobeline with a cough as the end point. They had from 30 to 40 per cent failures. The method was improved by Piccione and Boyd[18] who used a tickling sensation in the throat as an end point.

In 1935 Hitzig[19] used the injection of ether to measure the arm-to-lung circulation time. The detection of the ether in the expired air by the patient and often by the observer signals the end point. The lapse of time varies between 4 and 8 seconds.

In 1936 Goldberg[20] injected a solution of calcium gluconate and found that by using the sensation of heat on the tongue as an end point the arm-to-tongue circulation time averaged 12.4 seconds, varying between 10 and 16 seconds.

In 1936 Spier, Wright, and Saylor[21] injected a solution containing magnesium sulfate, calcium gluconate, sodium chloride, and copper sulfate. The arrival of the solution at various stations was signaled by a sensation of heat. They found the average circulation time of the arm to the tongue to be 14.6 seconds, to the hands 26 seconds, and to the feet 28 seconds.

In 1935 Zwillinger[22] suggested the use of magnesium sulfate as the test drug. In 1937 Neurath[23] employed this drug extensively, using the sensation of warmth in the head as the end point.

In 1939 Gubner, Schnur, and Crawford[24] used the inhalation of carbon dioxide to measure the circulation time from the pulmonary capillaries to the head. The end point was a feeling of warmth in the head and an acceleration and increased depth of respiration. This time ranged from 5 to 10 seconds.

In 1922 Koch[25] injected fluorescein to measure arm-to-arm time and found it to vary from 12 to 26 seconds.

Recently Fishbach[26] reported the arm-to-eye time estimated by the injection of sodium fluorescein to vary between 7 and 15.6 seconds. The end point is the appearance of a bright yellow fluorescence in the lower conjunctiva when viewed by ultraviolet light in a dark room.

A critical review of these methods strongly suggests the desirability of establishing criteria for the acceptance of any method. The most desirable method must conform to the following requirements:

1. The injected material must be nontoxic in the doses used.

2. The injection of a small quantity (1 c.c. or less) should be sufficient to give a sharp end point so that inaccuracies resulting from time loss during the injection be avoided.

3. The end point should be sharp and reliable.

4. The end point should be objective to eliminate the source of error inherent in the untrained observer and the possible sluggishness of mental reaction of the patient and to extend the applicability of the method to children, the uncooperative, and the comatose.

5. The minimum of apparatus in simple form is necessary to facilitate clinical use.

6. The agent used should be readily available.

The xanthines have long been known to be respiratory stimulants. The observation that the intravenous injection of one of them, aminophyllin, produced a sharp respiratory gasp suggested its use for the determination of arm-to-head circulation time.

METHOD

The patient should be lying flat on the back for the brief duration of the test. A No. 20 gauge needle is introduced into a vein in the antecubital fossa. The tourniquet is released and thirty seconds later 1 c.c. of aminophyllin (0.24 Gm.) is rapidly injected while a stop watch marks the moment of beginning of the injection. The awaited end point is a marked increase in the depth of inspiration which in many cases amounts to a gasp. While this occurs in every instance, in some it may be preceded by swallowing movements, a change in facial expression resulting from subjective sensations, or sharp catch during expiration. The first observable change is taken as the end point.

RESULTS AND DISCUSSION

Ninety-two patients at various stages in the postoperative state were subjected to this test. This included males and females ranging in age from 12 to 82 years.

The characteristic reaction of the patient was a transient dizziness, flushing, and a hyperpnea. In no case did this fail to pass off in a few minutes, and no other untoward effects were observed.

The circulation times varied from 7.1 to 20.4 seconds, averaging 12.4 seconds.

This test under the limited conditions of trial here described has been perfectly safe. No special apparatus is required.

The preparation is available in 2 c.c. ampules previously recommended for intramuscular use. Although as little as 0.25 c.c. is frequently sufficient to give an end point as determined by preliminary studies, the larger dose of 1 c.c. is preferable because it always gives the response, and yet it is still a small enough quantity to be quickly injectible.

Confirmation of the respiratory response to the intravenous injection of aminophyllin is found in the report of Sperling, Weisman, and Papermaster[27] who used it postoperatively in the hope of diminishing atelectasis and pneumonia. They graphically demonstrated the response by recording the respiratory excursion with the ordinary basal metabolism machine. In a study of twenty-two patients they found "an immediate increase of the depth of respiration averaging 51 per cent."

It is an objective method with a sharp end point. In the seventy-two instances in which it was used by us, in only one case was there any difficulty in recognizing the end point.

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CHRONIC HEMOLYTIC ANEMIA WITH PAROXYSMAL NOCTURNAL HEMOGLOBINURIA*

Case Report With Experimental Studies

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CHRONIC hemolytic anemia with the accompanying features of hemoglobinemia and paroxysmal nocturnal hemoglobinuria is characterized by constant and excessive intravascular red blood cell destruction with exacerbations that are provoked by sleep, whether during the day or night. This syndrome was first described by Marchiafava and Nazari in 1911,[1] discussed again in 1928 and 1931 by Marchiafava,[2] and at length in 1931 by Micheli,[3] and is frequently called the Marchiafava-Micheli syndrome. Approximately 40 cases have been reported.

This disease has two fundamental features; first, the intravascular destruction of the erythrocytes, mainly during sleep, and secondly, the paroxysmal appearance of hemoglobin in the urine.

The agent producing this type of intravascular hemolysis remains unknown. The presence of hemoglobin in the blood plasma between the periods of hemoglobinuria, the anemia, if the regenerative efforts of the bone marrow do not fully compensate for the excessive hemolysis, and the persistent reticulocytosis are the signs of constant erythrocytic destruction. There is no familial tendency and no relation to food, drink, posture, drugs, or geographical distribution has been noted. Prolonged exposure to sun, severe chilling, and physical effort cause no exacerbations. About 70 per cent of the cases have been in males, with an average age incidence between 20 and 40 years. In the reported cases the disease has been fatal, usually within two to five years, and all therapeutic procedures, including splenectomy, have been unsuccessful. Mackenzie,[4] in discussing the "march" type of hemoglobinuria observed in soldiers after long marches, or occasionally in persons after severe physical effort, suggested that the increased blood levels of carbon dioxide during exercise may act as a hemolytic agent.

Ham,[5] who has studied this disease very thoroughly in five cases, concluded that the defect was present in the red blood cells, and not in the blood serum, and that the erythrocytes were abnormally sensitive, even within physiologic range, to normal variations of the blood pH toward the acid side during sleep. Ham, as well as Buell and Mettier,[6] demonstrated that the essential factor for hemolysis in the serum of these patients is destroyed by heating to 56° C. and is not restored by the addition of complement. Buell and Mettier,[6] after carrying out studies on the hydrogen-ion concentration, concluded that the fundamental disturbance appears to be in the red blood cells which become sensitized to a lysin normally present in human serum.

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In view of the above concept, alkali salts have been administered by various investigators in an effort to render the blood pH more alkaline, but this has resulted only in temporary improvement, followed by an increase in the degree of hemoglobinemia and hemoglobinuria. Likewise, in the case reported here, this has proved to be an unsatisfactory procedure, since the prolonged administration of alkali salts was followed by a marked accentuation of the hemoglobinemia and hemoglobinuria.

The mechanism responsible for hemoglobin in the urine includes two major factors. The first, as stated before, is the intravascular destruction of the erythrocytes in excess of the reticulo-endothelial capacity to convert the released oxyhemoglobin into bilirubin, thereby resulting in free hemoglobin being dissolved in the blood plasma; and secondly, the renal threshold for hemoglobin. The latter is based upon the functional capacity of the nephron unit to pass the large hemoglobin molecule when an adequate plasma concentration is attained. An explanation of this threshold might be found in Whipple's[7] "glomerular threshold" concept whereby it is assumed that hemoglobin passes through the glomerular filter and is rapidly reabsorbed by the tubular epithelium. As the rate of filtration through the glomeruli exceeds the rate of absorption by the tubules, hemoglobin appears in the urine. It is generally conceded that hemoglobin passes the glomerular filter. Bieter[8] has shown that the aglomerular nephron does not excrete hemoglobin even after being damaged with bichloride of mercury. As yet it is not known how the large hemoglobin molecule with a molecular weight of 67,800 passes the glomerular filter when the molecule of serum albumin with a smaller molecular weight of 67,000 fails to pass it. Bieter,[8] on the basis of variation in size of the glomerular membrane pores, has estimated that 3 per cent of these are of sufficient size for the passage of the hemoglobin molecule. Yet hemoglobin has been observed in the urine of these patients without albumin.

Factors other than molecular size may also be involved in the passage of proteins through the glomerular membrane. Risse[9] maintains that the electrical charge of the protein ion and the glomerular membrane is concerned. Webster[10] and his associates studied the physicochemical factors controlling the passage of proteins across the glomerular membrane and found that the elimination of dissolved hemoglobin by the perfused frog kidney was greatly increased at the acid side (pH 5.5) and was much less at the alkaline side (pH 7.8) of the pH range studied.

If the permeability of the glomerular membrane is altered on exposure to high concentrations of hemoglobin, albuminuria theoretically, at least, should accompany hemoglobinuria, yet it does not always do so. Different threshold values have been observed in human beings and in dogs. Monke and Yuile[11] have determined the average value for the initial rate of tubular absorption in dogs to be between 2 and 3 mg. per minute, with diminishing values to less than 1 mg. per minute after the threshold has been lowered by daily injections of hemoglobin sufficient to cause gross hemoglobinuria.

In summary then, the mechanism may be a combination of altered glomerular permeability and tubular reabsorption, but at the present time most of the evidence indicates that tubular reabsorption capacity is entirely responsible for the phenomenon and hemoglobin will appear in the urine when the plasma concentration attains a level of 130 to 150 mg. per 100 c.c.

CASE REPORT

Present Illness.—The patient dates the onset of his weakness from July, 1935, following the extraction of a tooth, at which time no unusual quantities of blood were lost. Because of this weakness he consulted a physician who informed him that he had pernicious anemia and advised treatment. Liver injections were begun. At about this same time he began to have indigestion which he described as a postprandial epigastric fullness, some abdominal gaseous distention, and a burning sensation which extended from the pharynx to the epigastrium. Some of his friends first commented on his pale appearance during 1934. After a vacation he returned to work in August, 1935, and continued his duties until hospital admission, except for an occasional day or two when he remained at home because of weakness. He was treated constantly by his physician with weekly injections of liver extract, and sometimes from two to three injections per week from 1935 until the first half of 1939. During 1935 he first noticed that his morning specimen of urine was frequently dark red to almost black in color. This continued to a variable degree until June, 1939, when the day urine specimens were sometimes dark and the morning specimen always red to black.

His anemia became so severe in 1938 that he was given his first transfusion. A total of six were given during this year and the first half of 1939. Each time he would return to his work after a few days of rest at home.

Following a transfusion he had a mild chill and noticed that his urine was much darker, but this was not disturbing for he had noticed the same type of discoloration on former occasions. Also, after another citrate transfusion of 500 c.c. of whole blood in November, a severe generalized urticaria and edema of the entire body occurred. These subsided following the administration of adrenalin subcutaneously, ephedrine sulfate by mouth, and several days of bed rest.

Past History.—Childhood illnesses included whooping cough and pneumonia at one year of age, and later mumps and measles without complications. During childhood he played with other children without fatigue. In 1928 he became employed in the box department of a paper company and remained at his job until May 1, 1935, when he was transferred to the stereotype department. This work was done in a poorly ventilated room and consisted of melting the metal composition at very high temperatures and pouring the type.

System Review: The cardiorespiratory, gastrointestinal, and genitourinary systems were negative except as mentioned above. There was no evidence of food allergy. He has never had areas of anesthesia, paresthesia, or hyperesthesia. There was a gradual loss of weight from 160 pounds in 1935 to 140 pounds in 1939.

Family History: His mother died in her late thirties following complications of tubal pregnancy; his father died at 50 years of age with Bright's disease; and one brother and one sister died in infancy from unknown causes. One brother is living and well. Deaths on both sides of the family have usually been due to kidney and heart disease.

Marital History: He married in April, 1935. His wife had always been in good health but never became pregnant as precautionary measures were taken.

Physical Examination.—He was a well developed and moderately well nourished white male, about 27 years of age, with a distinct pallor but no excessive skin pigmentation. His temperature was 98° F., pulse rate 88, respiration normal, and blood pressure 154/74. The positive physical findings were confined to a slight icterus of the sclera and a marked pallor of the conjunctiva and the oral mucous membrane. The gums around the lower central incisors were diseased with pyorrhea and were beginning to recede from the teeth. There was no evidence of cardiac enlargement. Soft blowing systolic murmurs were present at the mitral, aortic, and pulmonic areas. The aortic second sound was loud and snapping and greater than the pulmonic second sound. The liver was normal in size, the spleen was not palpable nor was its area of dullness increased. The reflexes were physiologic and lymphadenopathy was confined to a few small shotty glands in the inguinal region. The nail beds were very pale. The weight was 145 pounds.

Laboratory Findings.—

Erythrocytes	2,170,000 per cubic millimeter
Hemoglobin	7.7 Gm. (50 per cent)

Leucocytes	4,650 per cubic millimeter
Differential count	
Mature segmenters	54 per cent
Bands	14
Eosinophiles	1
Lymphocytes	25
Monocytes	6
Reticulocytes	20 per cent
Platelets	150,000 per cubic millimeter
Clot retraction time	Normal
Bleeding time	Normal
Coagulation time	Normal
Blood group	O (International)
Kahn	Negative
Volume index	1.01
Sedimentation rate	Westergren—70 mm. at one hour
	Wintrobe (corrected)—46 mm. at one hour

The stained smear revealed the erythrocytes to have an average or slightly larger than normal diameter, giving the appearance of slight macrocytosis with a slight degree of hyperchromia accompanied by a moderate variation in size and shape of the red blood cells. There were no abnormal leucocytes, and the platelets were normal in number and appearance. Thick- and thin-drop preparations for malarial parasites were negative. Blood examination during the night for filaria was negative. The van den Bergh reaction was indirect with 1.7 mg. of bilirubin per 100 c.c. of serum.

Icterus index	17	
Blood cultures	Anaerobic, aerobic, and under 10 per cent carbon dioxide were negative	
Blood chemistry		
Nonprotein nitrogen	32 mg. per 100 c.c.	
Creatinine	1.3 mg. per 100 c.c.	
Sugar (fasting)	106 mg. per 100 c.c.	
Calcium	11.6 mg. per 100 c.c.	
Phosphorus	3.6 mg. per 100 c.c.	
Chlorides (whole blood)	480 mg. per 100 c.c.	
Sodium (serum)	376 mg. per 100 c.c.	
Potassium (serum)	20 mg. per 100 c.c.	
Cholesterol	126 mg. per 100 c.c.	
Total protein	6.9 Gm.	
Albumin	4.0 Gm.	
Globulin	2.9 Gm.	
Fragility test	Control	Patient
Beginning hemolysis	42	42
Complete hemolysis	32	32

Spectroscopic examination of plasma and serum revealed absorption bands of oxyhemoglobin. No porphyrin or other abnormal bands were present.

Morning urine	
Color	Dark red
Specific gravity	1.025
Albumin	Trace
Acetone	Negative
Diacetic acid	Negative
Benzidine test	4 plus
Bile	Negative

Urobilinogen	Slightly increased. Morning specimen positive in 1:30 and day specimen 1:20 dilutions
Microscopic	Innumerable triple phosphate and amorphous phosphate crystals. No red cells
Spectroscopic	Revealed absorption bands of oxyhemoglobin. No prophyrin absorption bands were present

All specimens of urine voided during the day and night were saved. Without exception those voided during the night and on rising were red to black in color without the presence of red blood cells and gave a strongly positive benzidine reaction. The patient's sleeping habits were reversed so that he slept during the day and stayed up at night. Still the red to black urine followed sleep, whether during the day or night.

Donath-Landsteiner Chilling Test	Negative
Urine examination for lead	0.035 mg. per 100 c.c.—patient 0.035 mg. per 100 c.c.—control
Cevitamic acid content of urine per twenty-four hours	20 mg.

Cystoscopic examination and retrograde pyelograms were normal.

The kidneys excreted 67 per cent of the phenolsulfonphthalein dye in one hour after it was given.

Skin biopsy (stained with Prussian blue)	Negative for hemosiderin
Basal metabolic rate	Plus 8
Stool examination	There were no parasites, ova, or abnormal bacterial flora on culture studies.
Gastric analysis	
Fasting specimen	35 degrees free acid
30 minutes after Ewald meal	52 degrees free acid
Lactic acid	Negative
Blood and other abnormal contents	Negative

Skin tests for food sensitivity: Positive reactions were obtained for shrimp, scallop, crab, cherry, prune, cocoa, banana, carrots, celery, mushroom, cranberry, and dates.

Diagnosis.—The diagnosis of paroxysmal nocturnal hemoglobinuria was based on the nocturnal exacerbations of hemoglobinuria associated with the anemia, reticulocytosis, and hemoglobinemia. Paroxysmal cold hemoglobinuria, the most common of the paroxysmal types, was excluded since there was no history or clinical evidence of syphilis; the Wassermann, Kahn, and Donath-Landsteiner reactions were negative, and exacerbations of hemoglobinuria were not provoked by chilling.

Paroxysmal march hemoglobinuria was not considered, since it is usually observed in soldiers following long marches and is directly related in other isolated instances to physical effort. In the march type there is no evidence of a constant intravascular erythrocytic destruction, and even though there is a transient hemoglobinemia there is no sustained anemia, reticulocytosis, or icterus.

Favism,[12] a form of allergic paroxysmal hemoglobinuria, was excluded by the elimination without beneficial results of those foods to which the patient gave positive skin reactions.

EXPERIMENTAL STUDIES

Blood pH Determinations.—Procedure: Venous blood was drawn from the anterior cubital vein into a dry syringe without the use of a tourniquet. Heparin was used as the anticoagulant, and no suction was applied to the plunger of the syringe during the withdrawal of the blood. The Beckman glass electrode potentiometer was used to obtain the hydrogen-ion determination. After withdrawal the blood was immediately transferred to the cup of the instrument, the glass electrode was immersed, and the pH was determined. Controls were done on normal persons using the same instrument and technique. All blood specimens taken during waking hours were obtained after two or more hours of bed rest. Blood samples were taken immediately after being awakened in the morning, in the evening, and during the night hours (see Table I). The patient and control were awakened from sleep for the latter. In one instance the sleeping habits of the patient and control were reversed and determinations were made.

TABLE I

DATE	HOUR	pH OF VENOUS BLOOD		PATIENT'S PLASMA Hb.	COLOR OF PATIENT'S URINE	BENZIDINE REACTION OF URINE
		PATIENT	CONTROL	CONC. IN MG./100 C.C.*		
12/ 7/39	7:00 P.M. (up all day)	7:37	7:39	80	Yellow	–
12/ 7/39	12:00 P.M. (awakened from sleep)	7:40	7:43	180	Dark amber	+
12/ 8/39	7:00 P.M. (up all day)	7:29	7:32	110	Amber	–
12/ 9/39	7:30 A.M. (awakened)	7:33	7:30	280	Black	++++
12/10/39	12:00 P.M. (awakened)	7:43	7:24	270	Black	+++
12/11/39	7:00 P.M. (up all day)	7.43	7:32	120	Yellow	–
12/12/39	8:00 P.M. (up all day)	7:30	7:35	95	Yellow	–
12/13/39	8:00 A.M. (up all night)	7:39	7:35	105	Yellow	–
12/13/39	5:00 P.M. (slept during day)	7:39	7:31	185	Dark red	++
12/14/39	7: A.M. (up all night)	7:35	7:30	100	Yellow	–
12/14/39	1:30 P.M. (awakened from sleep)	7:45	7:42	181	Dark red	++

*Blood plasma of control contained no hemoglobin.

No consistent variations of the blood hydrogen-ion concentration, as indicated by blood pH determinations, could be shown on the specimen of blood taken after the patient was up the entire day or on the specimen obtained when the patient was awakened from sleep, whether during the night or at the usual

hour of rising. The mean average for pH determinations obtained after being awake was 7.35 and that after being asleep was 7.40; whereas that of the control subject during waking hours was 7.33 and after being asleep was 7.34.

The observations on this patient and the control showed very little difference in the pH of the blood after being awake and on being awakened from sleep. The mean values obtained after sleep are slightly more alkaline in both the patient and the control. In this case, there does not appear to be a definite relationship between the hydrogen-ion concentration of the plasma and the nocturnal exacerbations of the intravascular red blood cell destruction.

As Ham has pointed out,[5] it is possible that the deeper portions of the body have a greater variation of the blood pH, within the normal range, on the physiologic basis of a slowed blood stream and shallow respiration during sleep. However, it would appear that the slowed blood stream and shallow respirations during sleep are evidences of a lowered metabolism accompanying a decrease in the physiologic demands of the respiratory and circulatory systems at this time. Furthermore, the reserves of the blood buffer system should be adequate to compensate for slight variations of carbon dioxide during sleep without disturbances of the blood pH if it is capable of maintaining the balance during exercise.

Plasma Hemoglobin Determinations.—The blood plasma was utilized for quantitative plasma hemoglobin determinations by using the Sheard-Sanford photelometer. Comparatively large volumes of plasma (from 2 to 3 c.c.) were necessary to obtain accurate and consistent readings. A voided specimen of urine was obtained from the patient each time blood samples were drawn. The color and the benzidine reaction of each are recorded in Table I.

The plasma hemoglobin concentration obtained during different times of the day and night varied from 80 to 280 mg. per 100 c.c. of plasma. The lower levels occurred after being awake for a number of hours and the higher levels following sleep.

The urine specimens varied in color and intensity of the benzidine reaction. All the blood plasma concentrations between 80 and 120 mg. per 100 c.c. had an accompanying yellow or amber urine which did not contain hemoglobin, whereas the levels from 180 to 280 mg. per 100 c.c. had a urine red to black in color and a marked variation in the intensity of the benzidine reactions. No blood levels were obtained between 120 and 180 mg. per 100 c.c.

The urines were usually red and gave a 2 plus benzidine reaction when the plasma levels of hemoglobin were 180 mg. per 100 c.c. This would indicate, as near as can be determined from voided specimens, that the renal threshold for hemoglobin in this person is well below 180 mg. per 100 c.c., and above 120 mg. per 100 c.c.

Effect of Sympathetic Stimulant.—As sleep in this disease is accompanied by exacerbations of intravascular erythrocytic destruction, the physiologic features concerning the state of sleep were considered. The cause and nature of sleep remain obscure. Many theories have been advanced. Among the more widely accepted is that of Hess,[13, 14] who after extensive investigation concluded that sleep was a parasympathetic function. He pointed out in support of his theory that the constriction of the pupils, the bradycardia, and the vasodilatation

accompanying sleep are parasympathetic functions and respond to parasympathetic stimulants. On this basis, a series of experimental studies were carried out on this patient in an effort to determine the effects of sympathetic stimulants.

TABLE II

DATE	TIME AND AMOUNT DRUG GIVEN	URINE COLOR AND TIME VOIDED		COLOR OF SPECIMEN REMAINDER OF DAY	R.B.C.	HB. (GM.)	RETIC. (%)
1/25/40	None	Red 2:30 A.M. Benzidine positive	Black 8:00 A.M. Benzidine positive	Yellow to amber with occasional benzidine positive	2,100,000	7.8	18
1/26/40	0.5 c.c. adrenalin 9:00 P.M. and 1:00 A.M.	Light amber 1:00 A.M. Benzidine positive	Amber 7:00 A.M. Benzidine positive	Same	2,100,000	7.8	14
1/27/40	0.5 c.c. adrenalin 9:00 P.M. 2:00 A.M.	Yellow 2:00 A.M. Benzidine positive	Light red 7:00 A.M. Benzidine positive	Same	2,100,000	7.8	14
1/28/40 through 1/31/42	0.5 c.c. adrenalin 9:00 P.M. 2:00 A.M.	Dark amber 2:00 A.M. Benzidine positive	Amber 8:00 A.M. Benzidine positive	Same	2,260,000 2,240,000 2,470,000	8.3 8.5 8.5	12 12 14
2/ 1/40 2/ 2/40	Adrenalin omitted	Light red 4:00 A.M. Benzidine strongly positive	Light red 8:00 A.M. Benzidine strongly positive	Same	2,300,000	8.3	14
2/ 3/40 through 2/14/40	0.5 c.c. adrenalin 9:00 P.M. 2:00 A.M.	Yellow to amber 2:00 A.M. Occasional benzidine positive	Amber 8:00 A.M. Occasional positive benzidine	Same	2,250,000	8.6	14

The first was adrenalin. One-half cubic centimeter doses of adrenalin in oil (Parke, Davis & Co., 1:1,000 concentration) were administered subcutaneously at 9:00 P.M. and 3:00 A.M. each night (Table II). The date (Jan. 25, 1940) listed on Table II as having red to black urine after sleep is typical of the nocturnal hemoglobinuria occurring at that time. On the nights following the subcutaneous administration of adrenalin only an occasional urine contained sufficient hemoglobin to cause red discoloration. The injections were omitted on the nights Feb. 1, 1940, and Feb. 2, 1940, and the urines again were red with hemoglobin following sleep. The administration of adrenalin was resumed, and the urines returned to a yellow or amber color with only an occasional morning specimen having a positive benzidine reaction. The red blood cell count, hemoglobin, and degree of reticulocytosis were not appreciably altered during this time. The erythrocyte count increased about 400,000 cells per cubic millimeter and did not increase further even though the degree of nocturnal hemoglobinuria had decreased and the patient felt much better. The whole blood hemoglobin increased only slightly while the regenerative activity, as indicated by the degree of reticulocytosis, remained constant.

Synthetic suprarenin was then substituted for the aqueous extract of the suprarenal gland from Feb. 14, 1940, through Feb. 19, 1940. Beginning Feb. 16, 1942, the morning urines varied from a dark amber to very light red color, and all specimens after sleep gave a strongly positive benzidine reaction. During this period the red blood cell count remained at 2,300,000 cells per cubic millimeter.

All medication was discontinued on Feb. 19, 1940. After several days an occasional specimen voided during the night and the morning specimen of urine varied from a light to dark red color. Curiously enough, the synthetic suprarenin, in the same dosage and concentrations, did not bring about the same improvement in the hemoglobinuria as the aqueous extract of the medullary portion of the suprarenal gland. This would suggest the presence of additional beneficial agents in the natural extract other than adrenalin.

Effect of Parasympathetic Stimulants.—Since a sympathetic stimulant, adrenalin, had improved the state of hemoglobinuria and hemoglobinemia without appreciably altering the status of the blood, it seemed worth while to determine the effect, if any, of parasympathetic stimulants.

As an accentuation of the hemolysis and subsequent exacerbation of hemoglobinuria seemed possible, small doses of prostigmine (1 c.c. of a 1:4,000 solution) were given subcutaneously at the usual hours of 9:00 P.M. and 3:00 A.M. All urine specimens became normal in color, with only an occasional one giving a positive benzidine reaction. Injections of prostigmine were continued for seven nights. Then eserine sulfate in a dosage of 0.7 mg. subcutaneously at the same hours of 9:00 P.M. and 3:00 A.M. gave the same results. Pilocarpine hydrochloride in 3 mg. doses subcutaneously was started on March 11, 1940, and continued through March 26, 1940. All urines remained yellow to amber in color, free of hemoglobin, and the patient felt much better during this period of time. However, the red blood cell count never went above the level of 2,500,000 with 7.6 Gm. of hemoglobin. All urine specimens were of a normal color, with an occasional one giving a positive reaction for hemoglobin. Pilocarpine hydrochloride had been discontinued for twenty days before the urines voided during the night and on arising in the morning again became dark amber in color and consistently gave positive benzidine reactions. The red blood cell count at this time varied from 1,800,000 to 1,950,000, approximately 400,000 less than the levels obtained during the administration of parasympathetic stimulants.

Eschatin, the aqueous extract of the adrenal cortex prepared by Parke, Davis & Co., was started on April 15, 1940, in 0.5 c.c. doses subcutaneously at 9:00 P.M. and 3:00 A.M. and continued through April 25, 1942. This preparation was used because of the beneficial results obtained with the natural adrenalin, whereas the use of synthetically prepared epinephrine appeared to be of little or no value. The urines returned to a light amber or yellow color and were free of hemoglobin. After several days the red blood cell count increased to 2,300,000 cells per cubic millimeter but never attained higher levels.

Ten grams of sodium chloride were then administered orally in conjunction with the eschatin for a period of a week without beneficial or harmful effects. Then synthetic cortin, desoxycorticosterone, was substituted for eschatin during

a period of hemoglobinuria without alteration in the degree of the nocturnal hemoglobinuria.

The urines continued to be free of hemoglobin throughout the months of May and June, the erythrocyte count remained at about 2,200,000 cells per cubic millimeter, and the reticulocyte count varied from 10 to 20 per cent. At this time the patient was dismissed from the hospital, advised to limit his physical activities, and return for observation in the out-patient department.

Subsequent Course.—The patient did as he was advised and maintained an erythrocyte count of 2,100,000 with 21 per cent reticulocytes for the following six months. In January, 1941, he contracted influenza, and was hospitalized, and treated symptomatically. At that time the erythrocyte count was 1,680,000 per cubic millimeter with 12 per cent reticulocytes, but returned to 2,000,000 per cubic millimeter a few days after the infection disappeared. No hemoglobinuria occurred during this time. The temperature of the patient was always normal except during the upper respiratory infection. At this time the condition of the patient is about the same.

GENERAL DISCUSSION

The results of the agents employed in these studies are offered without an explanation of their mechanism. Papilian and Antonescu-Mazilu[15] recently investigated the antihemolytic action of adrenalin and showed that natural adrenalin inhibits the hemolytic effect of mercuric chloride and digitalis on erythrocytes in vitro and in animals. It is unusual that the aqueous extracts of the medullary and cortical portions of the adrenal gland will have similar effects on the nocturnal exacerbations of erythrocytic destruction. Yet the synthetic preparations of adrenalin and cortin were for the most part ineffective. This would suggest that the antihemolytic agent is neither adrenalin nor cortin, but another product which is present in both portions of the adrenal gland and is water soluble. Eschatin, the natural cortical extract, was more efficacious in decreasing the nocturnal exacerbations of hemolysis without having the stimulating side effects of adrenalin. Polycythemia[16] with characteristics of polycythemia vera has been observed in adrenal cortical tumors and basophilic adenomas of the pituitary.

The action of the parasympathetic stimulants is equally obscure. Stimulation of the parasympathetic system serves as an activator to the secretory epithelium of the gastrointestinal tract, its appendages, and the sweat glands of the integument. Perhaps the parasympathetic also serves to stimulate the adrenal gland to produce an antihemolytic agent or some product concerned with the development of erythrocytes that are more resistant to normal blood hemolysins.

These agents sufficiently decreased the hemolysis during the usual nocturnal exacerbations to lower the plasma hemoglobin below the renal threshold levels, thereby eliminating the symptom of hemoglobinuria which should be regarded as no more than a urinary manifestation of excessive intravascular red blood cell destruction.

It is not likely that the agents used in these studies acted favorably by altering the renal threshold for hemoglobin. Numerous physicochemical factors have been cited as controlling the passage of proteins across the glomerular membrane. Krogh[17] reviewed the evidence that the increased permeability of

the glomerular membrane often paralleled an increase in capillary diameter. Webster[10] and his associates, in their work on the perfusion of hemoglobin by the frog's kidney, found the glomerular membrane more permeable to hemoglobin and the capillary bed dilated at the acid end of the pH range. However, in the studies cited in this paper the hemoglobinuria was greatly lessened by capillary constrictors and disappeared with capillary dilators. Perhaps the improvement in the hemoglobinuria observed by Ham[5] following a limited administration of alkali salts and the relapse after excessive quantities results from an alteration in the physicochemical factors of the capillary bed of the glomeruli rather than the degree of intravascular red blood cell destruction.

It is impossible to know how much, if any, the course of the disease process has been altered in this patient by decreasing the nocturnal exacerbations of the hemolysis since the state of anemia has not been appreciably altered. Splenectomy has been reported to lessen the severity of the nocturnal hemoglobinuria, but the state of anemia and the downward course of the disease continued unaltered in these cases.

SUMMARY

1. A case of chronic hemolytic anemia with paroxysmal hemoglobinuria (Marchiafava-Micheli syndrome) is reported.

2. Reversal of the sleeping habits of the patient provoked hemoglobinuria during sleep, whether during day or night.

3. Determinations of the blood pH did not reveal abnormal variations in the hydrogen-ion concentration after the patient was awake all day or after sleeping all night. The mean average after sleep was slightly more alkaline (pH 7.4) than after being awake (pH 7.35).

4. The renal threshold for hemoglobin in this patient was between 120 and 180 mg. per 100 c.c.

5. The severity of nocturnal exacerbations of intravascular red blood cell destruction was markedly lessened by the use of adrenalin and eschatin, the aqueous extracts of the medullary and cortical portions of the suprarenal gland. Synthetic adrenalin and desoxycorticosterone did not alter the nocturnal exacerbations of hemoglobinuria.

6. The parasympathetic stimulants prostigmine, eserine sulfate, and pilocarpine hydrochloride were more efficacious than the suprarenal extracts in lessening the degree of the nocturnal red blood cell destruction and subsequent exacerbations of hemoglobinuria.

7. Pilocarpine hydrochloride was the most effective. On each occasion after its use hemoglobin disappeared from the urine for many days or even weeks.

8. The degree of anemia and reticulocytosis were not appreciably altered during the remissions of hemoglobinuria that were produced by the various agents employed in these experimental studies.

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VARIATIONS IN SUSCEPTIBILITY TO CINCHOPHEN AS OBSERVED IN ANIMALS WITH BILE FISTULAS*

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IT HAS been suggested by clinical and experimental observers that in some instances, if not all, the toxic manifestations of cinchophen are due to hypersensitivity.[1]

In the course of our studies of the elimination of cinchophen in the bile of dogs with chronic biliary fistulas, observations have been made which show that some dogs are more sensitive than others and that some dogs react more markedly to the first dose of the drug than to subsequent doses.

In this study we have directed our attention to disturbances of cholic acid output in the bile as well as to such symptoms as anorexia, vomiting, and diarrhea. It should be stated in advance that the animals used in these experiments represent a selected group to establish variations in susceptibility in the same animal and in different animals with a bile fistula. The seven susceptible ani-

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mals to be referred to come from a group of dogs with biliary fistulas which were given cinchophen several times over periods varying from two to thirty-three days.

METHODS

The animals had a chronic bile fistula of the Rous-McMaster type;[2] in addition, some had a duodenal fistula for the return of bile into the intestine. The animals weighed from 7 to 13 kg. All were allowed to recover from the operation and to reach a constant twenty-four-hour output of cholic acid on the standard diet. Numerous control tests on the diet with and without the return of bile had been conducted prior to the administration of cinchophen.

Three different types of experiments were performed. In those of Group I, the initial dose of cinchophen was given after a period of from three to four days, during which time the animal reached a basal control output of cholic acid on the diet fed twice daily without the return of bile. The cholic acid so formed comes from synthesis only. In those in Group II the daily ration of the standard diet was divided into three portions and fed every eight hours, with the bile secreted during the previous eight hours being returned to the duodenum during a period of about one hour after the meal. When the output of cholic acid had reached a relatively steady level, 0.333 Gm. of cinchophen was given with each meal. Thus the bile salts and cinchophen excreted in the bile (45 to 55 per cent of the oral dose) were subjected to three enterohepatic circulations daily. In the experiments in Group III, the animals, after being subjected to a basal control period on the diet without the return of bile, were given through the duodenal fistula the bile secreted during the preceding hour. At the start of the hourly return of bile, 0.5 Gm. of cinchophen was given with the meal, and another dose of 0.5 Gm. was given with the next meal twelve hours later, and thenceforth until 2 Gm. were given, the hourly return of bile being continued. Thus, the bile salts and cinchophen excreted in the bile were subjected to an enterohepatic circulation rather continuously as would occur in a patient without a gall bladder or with a nonfunctioning gall bladder.

The dose of cinchophen for the human adult varies from 2 to 5 Gm. daily, or from 30 to 70 mg. per kilogram. The average dose received by our animals was approximately 100 mg. per kilogram, or on the basis of body weight it was equivalent to twice the average dose given to man. A snow-white cinchophen powder* was used, which was odorless and completely soluble in alkaline aqueous solution.

RESULTS

Group I.—The four dogs referred to in Table I were allowed to reach their basal output of cholic acid on the diet. Then 0.5 Gm. of cinchophen was given with the meal every twelve hours for from three to five days.

One of the dogs B8, on the second day of medication (Table I) started and continued to vomit during the two-hour period preceding each meal. Since her stomach was about empty at the time, little food was lost. This is important because vomiting, particularly of food, decreases cholic acid output, since much

*Calco Chemical Co.

of the cholic acid is synthesized from the protein of the ingested food. After withdrawal of the cinchophen, the cholic acid output returned to the normal level within three days, during which time no vomiting occurred. Later, this animal was subjected several times to the same dose of cinchophen; no vomiting or anorexia and no decrease in cholic acid output occurred.

TABLE I

A DOSE OF 0.5 GM. OF CINCHOPHEN WAS GIVEN WITH THE MEALS TWICE DAILY WITHOUT THE RETURN OF BILE

DOG	DATE	BILE VOL. C.C.	TOTAL CHOLIC ACID (MG. PER 24 HR.)	REGIME
B8	Control	109	1,226	Control; average of six days
	2/24	234	1,508	0.5 Gm. cinchophen b.i.d.
	2/25	218	873	0.5 Gm. cinchophen b.i.d. Vomited a little
	2/26	232	684	0.5 Gm. cinchophen b.i.d. before meals
	2/27	202	738	0.5 Gm. cinchophen b.i.d.
C8	Control	136	1,675	Control; average of three days
	2/1	325	2,090	0.5 Gm. cinchophen b.i.d.
	2/2	336	1,686	0.5 Gm. cinchophen b.i.d.
	2/3	347	1,822	0.5 Gm. cinchophen b.i.d.
	2/4	310	1,792	0.5 Gm. cinchophen b.i.d.
C10	Control	150	1,740	Control; average of three days
	2/6	240	1,620	0.5 Gm. cinchophen b.i.d.
	2/7	234	1,640	0.5 Gm. cinchophen b.i.d.
	2/8	249	1,620	0.5 Gm. cinchophen b.i.d.
C7	Control	138	2,172	Control; average of three days unusually high output of cholic acid for control
	2/5	262	1,892	0.5 Gm. cinchophen b.i.d.
	2/6	203	1,386	0.5 Gm. cinchophen b.i.d.
	2/7	227	1,118	0.5 Gm. cinchophen b.i.d.
	2/8	336	1,688	0.5 Gm. cinchophen b.i.d.
	2/9	326	1,923	0.5 Gm. cinchophen b.i.d.
				The cinchophen was continued for twelve days with normal outputs of cholic acid, then withdrawn and started again three days later
	2/20	276	488	
	2/21	311	928	
				The cinchophen was discontinued for two weeks and then it was given again for three days with the following results:
	Control	181	2,280	Control period of 3 days
	3/5	299	2,590	0.5 Gm. cinchophen b.i.d.
	3/6	288	2,300	0.5 Gm. cinchophen b.i.d.
	3/7	247	1,950	0.5 Gm. cinchophen b.i.d.

Dog C7 (Table I) showed a similar depression of cholic acid output during the second and third days of cinchophen medication which returned to the "normal" level for this dog during the fifth day of medication. This animal manifested no anorexia or vomiting. The cinchophen was continued for a total of twelve days. Then the cinchophen after three days of withdrawal was resumed again. The cholic acid output markedly decreased, though there was no vomiting and no diarrhea. The drug was stopped for two weeks and then given for three days. Some, though not a significant, depression of the cholic acid output occurred on the third day. This dog represents a case in which the cinchophen caused a depression of cholic acid output each time it was given, more the second time it was given than the first. In addition, the cholic acid output was depressed without any apparent gastrointestinal symptoms.

Dogs C8 and C10 are included to illustrate that similar doses of cinchophen have no effect on appetite and cholic acid synthesis in some dogs.

Group II.—The animals in this group were fed every eight hours and received via the duodenal fistula after meals all the bile secreted during the previous eight hours, except 0.1 c.c. which was used for cholic acid determination. After the animals had reached a relatively steady state, 0.333 Gm. of cinchophen was given with each meal. The cinchophen excreted in the bile during eight hours after oral administration amounts to from 45 to 55 per cent of the oral dose. Consequently the liver of these dogs was exposed to about 1.5 Gm. of cinchophen each day. With the exception of the return of bile and the enterohepatic circulation of cinchophen, these experiments were analogous to those in Group I.

Dog C3 vomited the first day of cinchophen administration. After having received 1 Gm. of cinchophen, the medication was stopped, and then the bile return was stopped to ascertain whether the basal control output of cholic acid without the return of bile would be resumed. The data in Table II shows that although cinchophen caused vomiting, it had no definite effect on basal cholic acid synthesis on the diet, since the postcinchophen output of cholic acid was normal.

TABLE II

A DOSE OF 0.333 GM. OF CINCHOPHEN EVERY EIGHT HOURS WITH MEALS; THE BILE WAS RETURNED EVERY EIGHT HOURS JUST AFTER THE MEALS

DOG	DATE	BILE VOL. C.C.	TOTAL CHOLIC ACID (MG. PER 8 HR.)	REGIME
C3	Control	157	1,434	No bile returned, basal control
	8	218	5,002	Bile returned t.i.d.
	9	248	4,927	Bile returned t.i.d.
	10	353	6,332	Bile returned t.i.d., + 0.333 cinchophen; dog vomited once
	11	224	3,951	Bile returned first eight hours only
	12	251	1,475	No bile returned
C4	19	232	4,535	Bile returned t.i.d.
	20	246	5,511	Bile returned t.i.d.
	21	212	5,173	Bile returned t.i.d.
	23	268	2,847	Bile returned t.i.d., + 0.333 cinchophen; anorexia
	24	363	4,705	Bile returned t.i.d., + 0.333 cinchophen; anorexia
				(Other data omitted from Table. See text)
C2	8	212	5,856	Bile returned t.i.d.
	9	223	5,725	Bile returned t.i.d.
	10	365	7,895	Bile + 0.333 Gm. cinchophen t.i.d.
	11	340	6,557	Bile + 0.333 Gm. cinchophen t.i.d.
				(Other data omitted from Table. See text)

Dog C4 showed a significant (44 per cent) depression of cholic acid output during the first day of cinchophen administration. But on continued cinchophen administration, the output of cholic acid remained around 4.7 to 5.0 Gm. per day. The animal had a poor appetite but ingested the daily ration throughout, even during the first day when the output of cholic acid was depressed.

Dog C2 is included to illustrate an animal that showed no effect from the same dose of cinchophen.

Group III.—The animals in this test represent a group in which the bile was returned to the intestine hourly. 1. The animals were placed on the diet with

a meal every twelve hours without the return of bile (Column 1, Table III) until they reached a relatively steady output of cholic acid. 2. The bile secreted the previous hour was returned for a period of forty-eight hours, the cholic acid output for the forty-eight hours being determined by sampling the hourly specimens (Column 2, Table III). 3. Step 1, above, was repeated. 4. Step 2 was repeated except that 0.5 to 0.25 Gm. of cinchophen was given with each meal for forty-eight hours. 5. Step 1, above, was repeated.

TABLE III

A DOSE OF 0.25 OR 0.5 GM. OF CINCHOPHEN WAS GIVEN TWICE DAILY AND BILE WAS RETURNED HOURLY FOR FORTY-EIGHT HOURS; THE TOTAL CHOLIC ACID OUTPUT FOR SEVENTY-TWO HOURS IS GIVEN

DOG	TOTAL MG. CHOLIC ACID FOR 72 HOURS				REMARKS
	AVERAGE BEFORE TEST—NO BILE (24 HR.)	CONTROL TEST WITH BILE	CINCHOPHEN TEST WITH BILE	AFTER CINCOPHEN—NO BILE (24 HR.)	
A3	1,462	22,104	23,014	1,613	0.5 Gm. cinchophen b.i.d.
B3	1,547	--	9,109	910	0.5 Gm. cinchophen b.i.d.; dog died
D4	1,950	7,216	4,822	2,500	0.25 Gm. cinchophen b.i.d.
	1,630*	7,216	9,729	1,704	0.5 Gm. cinchophen b.i.d.; three weeks later
D6	1,023	8,577	5,731	1,252	0.5 Gm. cinchophen b.i.d.
	1,315*	8,577	8,997	950	0.25 Gm. cinchophen b.i.d.; two weeks later
	950*	8,577	7,416	1,060	0.5 Gm. cinchophen b.i.d.; three weeks later
D7	2,360	6,640	9,226	2,710	0.5 Gm. cinchophen b.i.d.

*These later tests are included to show the different cholic acid response to later doses of cinchophen.

Under the preceding plan the cinchophen excreted in the bile was subjected to repeated enterohepatic circulation. The dogs receiving 0.5 Gm. with each meal had their livers exposed to about 2 Gm. of cinchophen daily and the dog which received 0.25 Gm. with each meal had its liver exposed to about 1 Gm. daily. Thus it was possible to compare the effect of the cinchophen on bile formation when bile was being secreted at a rapid rate and the liver exposed to cinchophen constantly and to observe any after effects the cinchophen might have on basal cholic acid synthesis.

Dogs A3 and D7 were not affected by the cinchophen and are included to illustrate that some dogs show no ill effect from this dose and repeated enterohepatic circulation of cinchophen.

Dog B3, however, was markedly affected. It became sick during the second day and ate no food after the first day. The experiment was continued during the second day to ascertain if recovery would ensue. Cholic acid was completely depressed during the first twenty-four hours after cinchophen was stopped; that is, after four doses during forty-eight hours. After that, the cholic acid synthesis returned to about 50 per cent of the control level, in the presence of complete anorexia, and the animal died after having received only 2 Gm. of cinchophen. This animal died of some condition apparently caused by the cinchophen which was not significantly revealed by the mechanism re-

sponsible for cholic acid synthesis. Autopsy revealed only a severe gastroenteritis.

Dog D4 showed a 33 per cent depression of cholic acid output during the first period of administration of 0.5 Gm. cinchophen daily with return of bile and no depression of cholic acid synthesis after the cessation of cinchophen and the return of bile. However, at a subsequent period twice the dose of cinchophen had no apparent effect on the dog.

Dog D6 reacted like Dog D4 to the first period of administration of cinchophen and manifested no significant disturbance of cholic acid output during two subsequent periods, though a slight depression occurred with the second dose of 1 Gm. daily.

DISCUSSION

The variations in susceptibility of different animals to cinchophen has been observed by all who have used it for producing "peptic" ulcers in dogs and for studying its toxicity. We have observed the same variation in susceptibility in our dogs with bile fistulas.

We have also noted that the susceptibility is usually definitely less on the second and later administrations of the drug.

The symptoms in our dogs with bile fistulas were chiefly gastrointestinal (anorexia, vomiting, and diarrhea). This coincides with the observation made by Stalker, Bollman, and Mann,[3] Reid and Ivy,[4] and others,[5] who also observed that on continuous daily administration for several weeks the gastrointestinal symptoms frequently subsided after the first or second week, indicating an increase in alimentary tolerance. Death occurs early in those in which the gastrointestinal disturbance does not subside. Clinically it has been recommended that administration of the drug should cease if gastric distress occurs.[8]

The disturbance in cholic acid output observed in the animals reported in this paper can be accounted for by a gastrointestinal irritation, except in one instance out of seven. Any disturbance of protein digestion and absorption, since all the evidence indicates that cholic acid is synthesized from protein cleavage products, would decrease cholic acid production. Hepatitis of sufficient grade to cause a complete suppression of cholic acid synthesis occurred in only one dog (B3) and then only for one day, the dog succumbing to a severe gastroenteritis. It is possible, however, for cinchophen to disturb materially some important function of the liver which is not reflected by the rate of cholic acid synthesis. But, it is noteworthy that the rate of secretion of cholic acid was not materially affected, as shown by the dogs in Table III, when the liver is secreting bile salts at a level close to its maximum capacity.

We were disappointed in not observing a more definite and unequivocal hepatic injury as indicated by a reduction in cholic acid output in the susceptible dogs. The failure to obtain such evidence of hepatic injury, however, is consonant with the failure to find evidence of microscopic damage of the liver of dogs except when very large doses of cinchophen are given. No one has obtained any evidence of liver damage in the dog or any other animal unless more than 100 mg. per kilogram body weight of cinchophen has been administered.[1, 3, 7] Even then the gastroenteritis is so pronounced that one could

argue that the hepatic damage is secondary to the gastroenteritis. The only exception to the foregoing interpretation was Dog C7 (Table I), in which cholic acid synthesis was depressed definitely during two of three periods of cinchophen administration in the absence of gastrointestinal symptoms. Since diarrhea and vomiting did not occur in this animal, it appears as if the cinchophen was responsible for the depression of cholic acid synthesis. It is worthy of note that in this animal the bile was not being returned to the intestine.

SUMMARY

Seven dogs with bile fistulas with a duodenal fistula for the return of bile to the intestine, which were especially susceptible to the gastrointestinal irritation of cinchophen, were given doses of the drug (approximately 100 mg. per kilogram of body weight) equal to or twice the average human dose. Only seven of sixteen dogs with bile fistulas manifested the symptoms of anorexia, vomiting, or diarrhea under the conditions of our experiment. The output of cholic acid was studied under three conditions: (1) when the drug might affect cholic acid synthesis from a standard diet alone; (2) when the drug might affect both cholic acid synthesis and bile salt secretion as a result of the drug and bile salt excreted in the bile being subjected to three enterohepatic circuits daily; and (3) when the liver was continuously exposed to the cinchophen excreted in the bile by the hourly return of the bile. It was found that any changes in cholic acid synthesis and secretion observed in these susceptible dogs could be accounted for by the gastrointestinal irritation caused by the drug with but one exception. On repeated exposure of the dogs to the drug the gastrointestinal tract became more tolerant, and cholic acid synthesis and secretion were not disturbed.

The observations on the effect of cinchophen on cholic acid synthesis and secretion are consonant with the failure of others to find evidence of microscopic injury of the liver when similar doses of the drug were administered to dogs.[3] However, the one exception referred to shows that the drug may occasionally act on the liver to decrease cholic acid synthesis in the absence of objective symptoms of gastrointestinal irritation.

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RELATION BETWEEN AMOUNTS OF PERTUSSIS ANTIGEN INJECTED AND PRODUCTION OF AGGLUTININS

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I

THE freshly isolated disease-producing *Hemophilus pertussis* organisms called by us phase A[1, 2] and phase I by Leslie and Gardner[3] are used in active immunization in man. Although phase B organisms, termed by us the "nondisease-producing" stage (the "resting stage," the "fixed" bacilli, and phase IV by Leslie and Gardner), descend from the phase A types, they are said to be worthless as immunization agents. Guinea pigs can be protected against lethal doses of phase B organisms by the previous injection of phase B vaccine, but no protection is established against phase A organisms by actively immunizing these animals with phase A vaccines. Doses of the standardized phase B antigen (4 mg. of wet weighed organisms to 1 c.c.) used in previous experiments failed to produce any agglutinins against phase A bacilli.

The relative size of organisms in the two phase conditions is significant, the phase B type being 50 or even more times the size of phase A. One milligram of wet weighed phase A organisms contains definitely more bacteria than an equal amount of wet weighed phase B bacilli.

Years ago, Huenekens[4] stated that pertussis vaccines (phase B state) should be given in massive doses to secure results. Sauer[5] has recommended even larger doses of freshly isolated *H. pertussis* organisms (phase A state) for active immunization.

The size of the organism may conceivably be related to the ability to immunize and produce antibodies. Were the latter true, perhaps increased amounts of phase B organisms used as the stimulating antigen and injected into rabbits would produce a higher agglutinin titer in their blood serums against homologous and heterologous organisms than would smaller amounts.

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Methods and Materials.—No. 778 stock phase B strain culture (American Type Culture Collection) was grown in veal brain liquid medium.[6a] The viscid, sticky mass produced by the organisms was centrifuged down as much as possible, the supernate discarded, and the centrifugate dried and powdered in a lyophile apparatus. Some of the toxin previously described was nearly always present in this specimen.[6b] Twenty milligrams of the powder were added to each 1 c.c. of normal saline. Part of this 20 mg. suspension was diluted to make a standardized suspension of 4 mg. in 1 c.c. of normal saline. Eight rabbits were injected, four with the dose standardized at 4 mg. and four at 20 mg. The injections were given intravenously the same day and in the same amounts as previously described.[1]

The animals were bled ten days after the last injection of antigen. The blood was put in the refrigerator overnight, and the next day the blood serum was separated off by centrifugation. Agglutination tests were then done with twelve strains of *H. pertussis* (phase B state) that had become acclimated to veal brain agar. The type of agglutinin response has been described previously.[1]

There was an obvious increase in agglutinin titer in the blood serums of the four animals that received the larger doses of phase B organisms in toxin. The titer of agglutinins present in these latter animals was not tested to its end point in all instances, although with the smaller dosages an end zone was obtained.

The phase B antigen-antibody serum agglutinated Walker strain 4 plus at 1:640 and had no reaction at 1:1280. The exact end point between 1:640 and 1:1280 was not determined, but it was not pertinent since our objective was simply to determine if larger amounts of the same antigen, in this instance phase B organisms, in a small amount of toxin would cause an increase in agglutinin titer. The blood serum agglutinin titers of strains Walker, Nos. 13 and 15, were at least twice as high as that obtained with the lesser amount of antigen; those of Betty, Brescan, Nos. 5, 8, 12, 28, and 778, were at least four times as high; that of No. 23, at least eight times as high; and that of No. 9, sixteen times as high.

The results were clear-cut, and it could be concluded that when five times the volume of *H. pertussis* phase B organisms "in toxin" was used as an antigen, a relatively higher titer of agglutinating antibodies was produced than with one-fifth the volume of phase B organisms "in toxin."

The phase B serums were also tried with phase A organisms. The rabbit serums obtained by injecting 4 mg. of the antigen were consistently found wanting in agglutinin titer, as was found in a previous experiment where similar standardized doses of organisms were used as the antigen. The rabbit serum obtained by injecting 20 mg. of antigen showed suggestive plus-minus agglutination reactions in the 1:40 titer.

This might suggest the presence of specific agglutinins. However, although the phase A organisms used had all the qualities and characteristics of a phase A bacillus and had been carried on human blood potato agar, definite conclusions could not be reached, since all but one of these phase A organisms had been isolated several months before the experiment was done

and some of their phase A quality might have been converted into phase B quality, thus changing their reacting ability to specific serum. Especially was this suggestive since the one phase A organism isolated but twenty-six days prior to the experiment was not agglutinated in any dilution by phase B, 4 mg. or 20 mg. serums. Although phase B 20 mg. serums had been found to possess higher agglutinin titers than did phase B 4 mg. serums, it was still not known whether more antigen could produce a higher titer in the absence of what might be an enhancing "toxin."

This experiment with the dried antigen and toxin was repeated; the serums obtained were agglutinated against eight freshly isolated organisms that had been carried in their phase A state and the same organisms which had been acclimated to and grown on veal brain agar to a phase B state. The results were similar.

II

In the previous experiment, phase B organism antigen contained a "toxin" not present in the ordinary commercial vaccines. It was decided in so far as possible to work with pure organism antigens.

A relatively small amount of standardized phase A and B organisms had been used to inject the rabbit to produce agglutinins in the blood serums.

If the agglutinin titer of the serum were dependent upon the number of organisms injected, and if this number were increased more than in the last experiment and the "toxin" factor excluded, with the exception of that contained in the water of condensation, it might be that there would not only be an increase in blood serum agglutinin titer against the homologous phase B, but also against the heterologous phase A organisms.

Methods and Materials.—In the previous experiments, the organisms were grown in liquid media and "toxin" was produced. In this experiment, No. 778 *H. pertussis* stock phase B strain was grown on solid media brain veal agar for two days and mass production of "toxin" avoided. The growth was scraped off, wet weighed, and standardized at 4 mg. per cubic centimeter, 20 mg. per cubic centimeter, and 45 mg. per cubic centimeter. Two-tenths per cent formalin was added to the various standardized organism suspensions. The latter were shaken every day and kept in the refrigerator for seven days. Several rabbits weighing between 1 and 1½ pounds were bled and their serums tested for the presence of normal agglutinins, since occasionally agglutinins against *H. pertussis* are found in normal rabbits. When six animals had been obtained, the blood serums of which lacked normal agglutinins against either phase A or B organisms, two each were injected intravenously every four days with a different one of the three standardized suspensions, the dosages being 0.2 c.c., 0.5 c.c., 0.8 c.c., 1.2 c.c., 1.5 c.c., 1.8 c.c., 2.0 c.c., and 2.3 c.c. The rabbits were bled ten days after the last injection. The blood was left in the refrigerator overnight to allow clotting and was centrifuged the next morning until the serum was clear; it was then inactivated one hour at 56° C., and merthiolate preservative was added. Agglutination tests were done against homologous phase B and heterologous phase A organisms. There were three bleedings, on the thirty-second, forty-sixth, and sixtieth days after the last injection.

It was easy to inject rabbits intravenously with 4 mg. per cubic centimeter and 20 mg. per cubic centimeter of a standardized suspension of organisms, but difficulties were encountered with the 45 mg. per cubic centimeter dose. One of the rabbits (No. 149) injected with the latter amount died after the second dose of the antigen. One of the two animals substituted died after the first injection. Several other normal rabbits lacking agglutinins against phase A or phase B *H. pertussis* organisms were given the 45 mg. per cubic centimeter dose, so that even though the mortality rate might be high during the process of immunization, enough animals would remain alive at the end of the procedure to finish the experiment. Three of eight animals injected with the 45 mg. per cubic centimeter dose died during the course of injections.

Agglutination tests were set up against (1) twelve phase A organisms isolated and grown on human blood agar within a few months before the experiment was started and (2) six phase B organisms isolated several years prior and carried on veal brain agar. The first bleeding was done thirty-two days after the last antigen injection against many phase A organisms. There was an increase in agglutinins in the blood serums of three rabbits (Nos. 190, 120, and 147) injected with 45 mg. per cubic centimeter, and against a few phase A organisms in the serums of two others (Nos. 21 and 180). This ability to agglutinate had practically disappeared in the specimens of the second and third bleedings obtained forty-six and sixty-six days later. Thus, agglutinins against some phase A organisms can be produced by phase B bacilli, although to a titer of only 1:80. These agglutinins tend to disappear within a month or so after the last antigen injection.

These rabbits injected with 4 mg. per cubic centimeter or 45 mg. per cubic centimeter of phase B antigen showed good titer responses against their homologous phase B organisms in the first blood serums obtained. There might have been a slight increase in titer value with the 45 mg. per cubic centimeter antigen, but it was not too definite. There was an increase in the agglutinin titers against phase B organisms in the specimens of the second bleeding, which was about equal in all serums regardless of the amount of antigen stimulus, whether 4 mg. per cubic centimeter or 45 mg. per cubic centimeter. The agglutinin titers in the specimens of the third bleeding had decreased to approximately those of the first blood serums and were about the same no matter what the stimulus.

It could be concluded that there was an increase in agglutinins against phase A organisms found in the first blood serums of the animals injected with 45 mg. per cubic centimeter of phase B, a titer lost only sixteen days later; and there was a good and almost equal response to all phase B organisms, irrespective of the stimulating phase B antigen and the time of bleeding, the highest titer being found in the blood specimen withdrawn forty-six days after injection. This conclusion differs from that of Experiment I, but the previous results could be easily explained because of the differences in the stimulating antigen, since only organisms were used in this experiment, and in the former work the organisms were contained in toxin, which possibly enhanced their antigen effect.

III

Stimulation with more antigen made from phase B organisms did not seem to increase the titer production against phase B organisms. However, there did seem to be a slight increase against the heterologous phase A organisms, so that it was thought that the surviving animals of Experiment II should be stimulated further.

All five animals of Experiment II that had survived the dose of 45 mg. per cubic centimeter of the phase B antigen were injected weekly for ten weeks with 2 c.c. of phase B organisms standardized at 45 mg. per cubic centimeter. There was an eleven-day interval between the first injection in this series and the final bleeding of the last series. Two of the five animals died; each of the remaining three were bled three times—twelve, twenty-three, and forty-four days after the last injection.

Agglutination tests were done with the nine blood serums of the three animals against five phase A organisms (Nos. 1, 2, 6, 7, and 8) isolated several months previously, five phase A organisms (Nos. 3, 4, 5, 9, and 10) isolated within the previous month, and three phase B organisms. The titers of the blood serum against phase B organisms were as usual, while against some heterologous phase A organisms, they were at least 1:80.

In a collateral experiment, agglutination tests were done against three phase B organisms and ten organisms which had been carried on human blood agar for several years, organisms which were beginning to show some phase B qualities. The latter, though carried on blood agar, were agglutinated by rabbit blood serums, in most instances to a titer of at least 1:320, thus demonstrating some approach to a phase B state.

IV

It was difficult to secure enough freshly isolated organisms to start a simultaneous experiment with many organisms where all the experimental factors would be the same. Some organisms grow faster than others and, as in the last experiment, a month or more might elapse before an experiment can be started. Meanwhile, the organisms, although grown on blood, might be progressing to a phase B state so that the titer obtained by phase B organism antibody serum might represent a reaction against some B-ness which was beginning to appear with age in the phase A organisms. To settle this point, another set of experiments were done. This time organisms were isolated, grown a few times on human blood agar, frozen in a lyophile apparatus, and set aside until at least five strains had been collected. By this method, the phase A characteristics were neither altered nor lost, and the organisms remained in their originally isolated state. The method of procedure was the same as in Experiment III.

Here again it was demonstrated that the homologous phase B organisms can produce some agglutinins against the heterologous phase A bacilli. These reactions are noted in Chart 1. Of the three rabbits used, the agglutinin titers of the blood serums of Nos. 147 and 190 were the highest after the first bleeding, and those of rabbit No. 21 might have been considered the same or slightly higher after the second bleeding. No animal had much of an increase in phase A agglutinins after the third bleeding.

COMMENT

We still use the terms phase A and phase B and not phases I, II, III, and IV as do Leslie and Gardner.[3] This is not in agreement with Dozois, Flosdorf, and Kimball.[7] The latter, in their work, constructed certain artificial boundaries and attempted to demonstrate three phases of existence. Yet at the same time they presented data concerning certain strains in various of their phases, which, because they did not fall within the limits of their phase boundaries, were described as "unrepresentative" and atypical"—and this despite the fact that these very exceptions pointed to the existence of an imperceptibly graded transition between two extremes of statistical maxima and minima; namely,

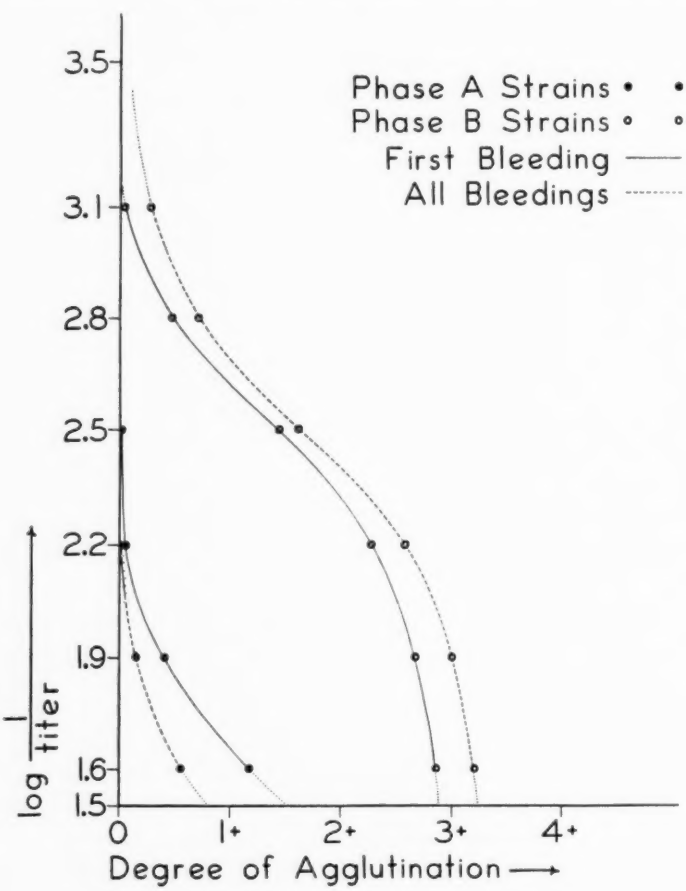

Chart 1.—Illustrating results of Experiment IV.

phases A and B (phase A, disease-producing stage; phase B, resting or non-disease-producing stage). Indeed, they explained away their "unrepresentative" strains by concluding that the phase relationships of *H. pertussis* are "more complex than was found by Leslie and Gardner," a conclusion with which we fully agree. In fact, the relationships are so complex that it would seem impractical to consider any other phases than the two previously mentioned.

Flosdorf and associates leave unexplained other data at variance with their theories, chiefly concerning the relationships between the cross-agglutination and cross-absorption characteristics of their phases I and III. They dismiss the fact "that the whole phase I organisms absorb phase III agglutinins" as "irrelevant, quantitatively, because other factors may be concerned in such reactions with particulate matter," a statement, which if true, should indicate that any agglutination or absorption test is subject to the same errors and is of proportionate relevance.

Flosdorf and associates did not agree with the premises on which we disputed Leslie and Gardner's assumptions as to phase existence. It has been shown, however, that *H. pertussis* organisms, if grown long enough on artificial media, with or without blood, gradually change their characteristics and in time reach a state in which all subcultured organisms, no matter what their original characteristics, acquire the ability to produce about the same degree of agglutinin titer in the blood serums of rabbits, have the same virulence, and appear morphologically the same as other phase B organisms carried on hemoglobin-free media. At the other extreme are the organisms freshly isolated from the human being ill with the disease, organisms which at the moment of isolation approach more or less the ideal opposite of the fixed stage. However, *H. pertussis* organisms seldom, if ever, attain either ideal extreme, for it has been shown here that by sufficiently intense programs of immunization it is actually possible to obtain cross-agglutinations between phases A and B. Would it not be theoretically possible to isolate any organism anywhere in its transition toward the resting stage at a time when it is losing some of the characteristics of phase I and acquiring some of the characteristics of phase IV and to immobolize it in this stage of its existence by a process of freezing and drying? This should not be impossible, since these authors themselves have demonstrated the existence of certainly more than four stages (phase variations).

It is our belief that the so-called phase II and phase III are only a few of the many possible "immobilizations of a phase" which might appear in the transition of organisms toward the resting or phase B state. The only two phases of which we ourselves are confident are (1) phase A, the disease-producing stage which in its freshly isolated state may have already started somewhat on its way toward the resting state and (2) phase B, the resting state.

Inferentially it could be said that if agglutinin production were a sina qua non of a good immunizing antigen, phase B organisms should be considered deficient as vaccines for human use.

SUMMARY

1. Rabbits given ten doses of phase B *H. pertussis* organisms standardized in toxin at 20 mg. per cubic centimeter have a higher agglutinin titer against phase B organisms than do the blood serums of those given the same number of doses of 4 mg. per cubic centimeter. Both antigens produced only a negligible titer of agglutinins against phase A organisms, the type which initiates the disease in man.

2. Four milligrams, 20 mg., and 45 mg. of phase B organisms for ten injections will produce nearly comparable agglutinin titers in rabbits against phase B organisms and some demonstrable but not marked agglutinins against heterologous phase A bacilli.

3. When rabbits are injected weekly over a long period with massive amounts of phase B organisms as the antigen, there is no increase in titer against the phase B organisms, but in some instances there may be an increase in the agglutinin titer against phase A bacilli—as high as 1:160.

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AN EXPERIMENTAL STUDY OF PHLEBITIS FOLLOWING VENOCLYSIS WITH GLUCOSE AND AMINO ACID SOLUTIONS

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ALTHOUGH injury to veins during intravenous injections is of frequent clinical occurrence, often producing thrombosis sufficient to preclude further use of the vessel, there have been few experimental studies of the various factors which influence its degree and severity. Presumably these influencing factors are numerous, some of which are as follows: The character of the solution (its chemical properties, its pH, and its osmotic pressure), the size of the vein injected, the rate and duration of injection, the trauma produced in introducing the needle, the trauma produced by the needle while resting in the vein, stasis resulting from the partial occlusion of the vein by the needle, and the previous state of health of the vein.

The present study was undertaken primarily to determine whether the presence of amino acids, especially in solutions of glucose, influences the degree of injury produced by intravenous injections as compared with simple solu-

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tions of glucose. As far as possible, all factors other than the character of the solution were kept constant.

METHOD

Dogs weighing approximately 10 kilograms were the experimental animals. The injections were carried out under nembutal anesthesia. Two pairs of veins were used; i.e., the large vein on the dorsum of both forelegs, known as the antebrachial cephalic, and the somewhat smaller, saphenous vein coursing along the inner aspect of both hind legs. The solution to be tested was administered on one side, the control solution simultaneously on the other side. Twenty-four hours later the two remaining veins were similarly injected. After a second twenty-four-hour period the dog was killed and the veins removed for section and study. Thus in most experiments each dog furnished two sets of veins, one showing changes twenty-four hours and one forty-eight hours after injection. The amount and rate of fluid injected were determined by the weight of the dog. Six cubic centimeters per kilogram per hour were accepted as fairly closely approximating the amounts used clinically. Because a three-hour injection usually produced definite changes with the first solutions used, this period was adopted. Twenty-gauge needles were used in all experiments, except in a few, which will be referred to below, when a twenty-five-gauge needle was used. The solutions were introduced at room temperature. The position of the point of the needle in the vein was marked by a silk suture placed in the skin just above it so that microscopic comparison could be made at the same distance from the point of injection. In early experiments sections were made longitudinally through the middle of the vein. In later experiments, cross-sections were taken two to three millimeters apart for use. The sections were stained with hematoxylin and eosin. In the study of many sections it was found that any injury observed was always greatest near the point where the end of the needle had rested. Thus, a section of vein taken about 1 cm. to either side of the needle point was used for the comparison.

Evaluation of Changes in the Veins.—In recording the changes in the veins, all observations were divided into those noted in the intima, those in the media, those in the adventitia, and also those within the lumen of the vessel. One of the earliest intimal changes observed in these sections was a vacuolation of the endothelial cells. This change might be observed in the absence of any other changes in the vessel wall. With further injury there occurred progressive cellular changes with ultimate death of the cells and destruction of the endothelial lining. Often, though not always, the denuded intima furnished a locus for the deposition of fibrin, and varying degrees of thrombus formation were observed. In the media the observed changes ranged from slight pyknosis of some of the nuclei to complete necrosis of the muscular wall. To establish a basis for comparing and evaluating the changes, they were classified as follows: (1) changes within the lumen, i.e., thrombosis; (2) changes in the intima, i.e., (a) endothelial vacuolation, (b) endothelial destruction; (3) changes in the media, i.e., (a) edema, (b) leucocytic infiltration, (c) hemorrhage, (d) necrotic changes; and (4) changes in the adventitia, i.e., (a) edema, (b) leucocytic infiltration, (c) hemorrhage. This classification is similar to though less detailed

TABLE I

EVALUATION OF THE SCORE INDICATING DEGREE OF DAMAGE TO VEIN DURING VENOCLYSIS

VEIN	SOLUTION	LUMEN	INTIMA		MEDIA			ADVENTITIA				HOURS	TOTAL SCORE
		THROMBOSIS	ENDOTHELIAL VACUOLIZATION	ENDOTHELIAL DESTRUCTION	EDEMA	HEMORRHAGE	LEUCOCYTIC INFILTRATION	NECROTIC CHANGES	EDEMA	HEMORRHAGE	LEUCOCYTIC INFILTRATION		
Right foreleg	11.4% G.	0	3	0	2	0	0	0	2	0	0	48	7
Left foreleg	5.0% A. 5.0% G.	2	3	2	2	0	2	2	3	0	0	48	16
Right hindleg	11.4% G.	2	3	2	1	0	2	2	3	0	2	24	17
Left hindleg	5.0% A. 5.0% G.	3	3	3	1	2	2	3	2	0	2	24	21

G., Glucose.
A., Amigen (hydrolyzed casein).

than that used by Ochsner and Garside[1] and by Ochsner and Mahorner[2] in their experiments on the effects of various sclerosing solutions used in the treatment of varicose veins. Each of the foregoing changes was recorded according to its severity. The absence of any change was rated as zero. Slight or early changes were rated as one, moderate changes as two, and advanced changes as three. By adding the ratings for each vein the final estimate or score of the severity of the phlebitis took the form of a total which was a rough numerical estimate of the degree of injury inflicted by the solution. While this type of scoring was based on the subjective evaluation of the degree of change seen, the observations were objective in so far as they were made with no knowledge as to which solution had been injected into the vein. When a number of the sections were rated separately by a second observer, the total scores obtained agreed rather well. A typical example of the method of scoring used is shown in Table I.

The amino acid solution tested in all experiments was made from an enzymatic hydrolysate of casein (Amigen*). The pH of this product in solution is approximately 4.5; when a more alkaline solution was tried, sodium hydroxide was added until the desired pH was reached. A 2.7 to 3.0 per cent concentration of the product in solution has been found to be isotonic with the blood; this is slightly greater than glucose. In comparing two solutions, each was isotonic.

FINDINGS

Eleven dogs were obtained for study, of which seven yielded four veins each, four but two veins each. In all, eighteen pairs of veins were observed. A summary of the scores is listed in Table II. The results are also described in detail as follows:

1. A 2½ per cent amigen solution in 10 per cent glucose was one of the first solutions tested against a control of 13 per cent glucose which has an osmotic pressure approximately equal to that of the amigen solution, as measured by its freezing point. In all three experiments (1, 1a, 2) no significant difference was found between the injury produced by the tested and the control solutions, although definite injury was produced in each case. The tendency to thrombus formation was frequent. When the injection of both test and control solutions was repeated twice in the same veins, thrombus formation and damage to the entire vein was marked. In all experiments injury produced by the same solutions was greater in the veins of the hind legs than in those of the forelegs. This was probably due to stasis because of the smaller caliber of the former; this factor is discussed below.

2. A solution containing 5 per cent amigen and 5 per cent glucose was tested against an 11.4 per cent glucose solution, the latter having an osmotic pressure equal to the former (Experiments 3 and 3a). In the forelegs the injury produced by the amino acid solution was greater than that caused by the glucose. In the veins of the hindlegs the damage produced by both solutions was severe, thrombosis being present in each case, and there was no significant difference between the two.

*Prepared by Mead Johnson & Co.

TABLE II

SUMMARY OF SCORES OBTAINED WITH VARIOUS SOLUTIONS

EXPERIMENT	VEIN	SOLUTION	HOURS	SCORE
1	R. F.	13% G.	24	12
	L. F.	2½% A.,10% G.	24	11
1a	R. H.	13% G.	48	20
	L. H.	2½% A.,10% G.	48	22
2	R. F.	2½% A.,10% G.	24	13
	L. F.	13% G.	24	19
3	R. F.	11.4% G.	48	7
	L. F.	5.0% A., 5% G.	48	16
3a	R. H.	11.4% G.	24	17
	L. H.	5.0% A., 5% G.	24	21
4	R. F.	5% A., 5% G.	24	5
	L. F.	8.8% A.	24	13
4a	R. H.	8.8% A.	48	9
	L. H.	5% A., 5% G.	48	14
5	R. F.	7.7% A.	24	18
	L. F.	10% G.	24	4
6	R. F.	7.7% A.	24	13
	L. F.	10% G.	24	15
6a	R. H.	7.7% A.	48	22
	L. H.	10% G.	48	18
7	R. F.	7.5% A., pH-7.3	48	4
	L. F.	7.5% A., pH-4.6	48	8
7a	R. H.	7.5% A., pH-4.6	24	20
	L. H.	7.5% A., pH-7.3	24	9
8	R. F.	7.5% A., pH-7.4	48	6
	L. F.	7.5% A., pH-4.6	48	13
9	R. F.	7.5% A., pH-4.6	24	20
	L. F.	7.5% A., pH-7.4	24	7

R. F., Right foreleg.
R. H., Right hindleg.
L. F., Left foreleg.
L. H., Left hindleg.
G., Glucose.
A., Amigen.

3. A solution containing 5 per cent amigen and 5 per cent glucose was tested against an isotonic amigen solution (8.8 per cent) and the score was even, i.e., in one experiment (Experiment 4) the former solution produced less damage, whereas in the other experiment (Experiment 4a) it produced more.

4. A 7.7 per cent amino acid solution, isotonic with a 10 per cent glucose solution, was tested against the latter (Experiments 5, 6, 6a). Thrombus formation was present in each case and the damage done to the vein wall was essentially the same for the two solutions in Experiments 6 and 6a, whereas in Experiment 5 the score for the amigen solution was eighteen against four for the glucose.

5. The significance of the pH of the amino acid solutions as a factor was also tested (Experiments 7, 7a, 8, and 9). In all experiments a 7.5 per cent solution neutralized to a pH of 7.4 produced less damage than the one left at pH 4.6; the difference was striking in three of the four experiments.

6. Early in these experiments, as noted above, it seemed trauma and stasis played an important part in the production of thrombosis. A small gauge needle, No. 25, was therefore tested against the larger standard 20-gauge needle. The same solution was, of course, injected through each needle at the same rate.

In one set of experiments (Experiments 10 and 10a) 2½ per cent amino acid in 10 per cent glucose was used. Here injury was greater in the veins injected with the larger (No. 20) gauge needle, this causing thrombus formation, whereas none was produced by the smaller (No. 25) needle. In the second pair of experiments (Experiments 11 and 11a) a 13 per cent glucose solution was used. No significant difference was found between the two needles. The different results obtained in these two sets of experiments may be due to a difference in the caliber of the veins of the dogs used.

COMMENT

Careful perusal of the scores listed in Tables II and III indicated the greater damage inflicted by the more hypertonic solutions, confirming many clinical observations. On the other hand, solutions containing 10 per cent glucose or less or its equivalent tend to produce less phlebitis. The presence of amino acids seems to have but slight effect on the amount of damage produced, although it is clear that the pH is an important factor. This probably explains the difference noted in Experiment 5, the only one in which there was a definitely increased phlebitis with amigen alone as compared with glucose alone. This effect is clearly demonstrated in Experiments 7, 7a, 8 and 9, in which the neutralized amigen provoked much less phlebitis than the unneutralized solution in all four sets of veins.

TABLE III

SUMMARY OF SCORES ATTAINED WITH DIFFERENT NEEDLES

EXPERIMENT	VEIN	NEEDLE	HOURS	SCORE	SOLUTION
10	R. F.	25	48	3	2½% Amigen
	L. F.	20	48	6	10% Glucose
10a	R. H.	20	24	17	2½% Amigen
	L. H.	25	24	4	10% Glucose
11	R. F.	20	24	13	13% Glucose
	L. F.	25	24	17	
11a	R. H.	20	48	26	13% Glucose
	L. H.	25	48	24	

The role of stasis and obstruction deserves some comment as already indicated. While a smaller needle should allow blood to flow around it, thus preventing stasis, this depends on the relative caliber of the lumen as compared with the gauge of the needle. It was our definite impression that this accounted for variations between the hind- and forelegs, although the objective evidence herein adduced does not permit such a conclusion. The considerations are of practical importance and deserve further study.

CONCLUSIONS

1. A method of scoring is proposed to measure the degree of phlebitis produced by intravenous injections.

2. Phlebitis is pronounced following a three-hour intravenous injection of a 2½ per cent amino acid-10 per cent glucose solution, due not to the presence of the amino acids but to the hypertonicity of the solution, inasmuch as the same changes are produced by isotonic (13 per cent) glucose solutions. Less

pronounced changes occur with more hypotonic solutions containing either amino acids or glucose or both.

3. Acid solutions tend to produce more phlebitis than neutral solutions as shown by comparing the damage produced by a 7.5 per cent solution of amigen at a pH of 4.6 with the same solution neutralized to pH 7.4.

4. The size of the needle used probably plays an important part in thrombus formation, although this factor is undoubtedly associated with the relative caliber of the veins.

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ERYTHROCYTE LONGEVITY IN DOGS AND RABBITS*

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NUMEROUS estimates have been made of the life duration of red blood corpuscles in various species. Ashby,[1] on the basis of agglutination experiments, has placed the life span of human transfused erythrocytes at thirty days or more. Eaton and Damren[2, 3] have studied the reticulocyte percentage peaks in rabbit blood following hemorrhage and have concluded that the life span of red cells in this species is about 8.5 days. Escobar and Baldwin[4] exposed animals to reduced atmospheric pressures to increase the number of red cells and then observed the time required for their return to normal at atmospheric pressure. This period of time was considered by the authors to represent the life duration of erythrocytes in the circulation. They thus obtained values of twelve to eighteen days in rats, sixteen to twenty-three days in dogs, and eighteen to thirty days in man. Hawkins and Whipple,[5] from a study of bile pigment output in dogs with biliary fistulas following hemorrhage or acetylphenylhydrazine, have reported that the life span of erythrocytes in this species is about 124 days.

The present communication presents a new approach to this subject through the depression of erythropoiesis by drugs in polycythemic animals. The experimental data for my calculations of erythrocyte longevity have been taken from previous papers[6-9] and other data of mine.

The method involves the production of experimental polycythemia in dogs or rabbits by daily exercise,[6] continued administration of cobalt chloride,[6-9] ex-

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posure to low atmospheric pressure[7] or daily ephedrine administration.[9] The high erythrocyte counts were then reduced by the additional daily administration of raw liver, choline hydrochloride, mecholyl, or other vasodilator drugs. It has been found that only a few days are required for these substances to reduce the red cell counts to or toward normal to points which seem to represent the maximal depression that these drugs are capable of producing. It is assumed that these drugs act as a "brake" on erythrocyte production until the cell count reaches normal, or near normal. If such is the case, the rate of depression of the polycythemia should establish the rate of natural death of the erythrocytes, and it is then possible to calculate the length of time which would be required for 100 per cent disappearance of the cells if the process were to continue. This calculation has been made from composite or average curves drawn from rabbit and dog experiments.

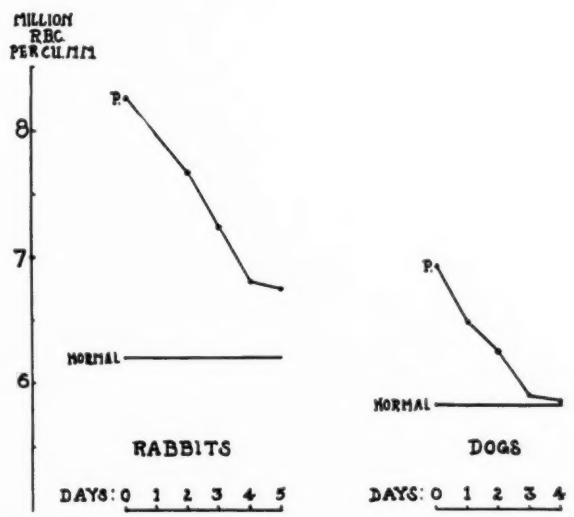

Fig. 1.—Average curves for rabbit and dog erythrocytes, showing the depression of experimental polycythemia by liver and various vasodilator drugs. *P* designates the average polycythemic level for each species.

RESULTS

Fig. 1 shows curves representing the depression by drugs of the average erythrocyte counts from polycythemic values to near normal in rabbits and dogs.

The average curve for rabbits is composed of twelve experiments on seven rabbits. It will be seen that four days were required for a practically maximal reduction of 17.8 per cent in the polycythemic cell count to occur. If this rate of reduction were to continue, all erythrocytes would disappear in 22.4 days.

The curve for dog erythrocytes represents thirty-three experiments on twenty dogs. It shows that a practically maximal reduction of 15 per cent of the polycythemic cell count occurred in three days. At this rate the last of the dog corpuscles would disappear in twenty days.

On the basis of these calculations, the minimal length of life of erythrocytes should be placed at twenty days in the dog and at about twenty-two days in the rabbit.

DISCUSSION

Our evidence for believing that the reductions of erythrocyte counts shown in Fig. 1 are due to a depression of erythropoiesis has been previously presented in articles cited above.[6-9] Since the depression occurs in spite of continuation of hemopoietic stimulating measures, and since the red cell count rises again to polycythemic values soon after cessation of administration of the depressant drugs, it seems justifiable to assume that the latter agents act as a "brake" on erythropoiesis. In some manner this brake seems to operate only until the erythrocyte number is reduced to a certain level which is not below the normal. We assume that the bone marrow still has an "excess momentum," so that when the "drug brake" is removed, an accelerated rate of hemopoiesis is able to cause a return of polycythemia in a few days.

Although it is recognized that the assumption that the rate of depression of polycythemia establishes the rate of natural death of erythrocytes is a broad one, it is perhaps permissible to say that it establishes the maximal rate of cell disappearance. Our calculated values for the life span of the erythrocyte should then be considered as minimum values.

CONCLUSIONS

On the basis of data on the rate of depression of experimental polycythemias by liver or vasodilator drugs, the *minimal average life duration* of erythrocytes has been calculated. The "life spans," thus obtained, are twenty days in the dog, and 22.4 days in the rabbit.

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CLINICAL CHEMISTRY

THE EFFECT OF CHANGE OF ALTITUDE ON THE BASAL METABOLISM OF HUMAN SUBJECTS*

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IN A study of the basal metabolism of the children of the Child Research Council at Denver, sufficient data have been accumulated to establish normal values for different age groups from the third through the sixteenth year. That portion of the data obtained prior to April, 1936, for children between the ages of 2 and 13 years has been reported by Lewis, Kinsman, and Iliff.[1] Since the publication of this study, pediatricians and investigators in the field of energy metabolism have asked from time to time whether the normal values of basal metabolism thus established at an altitude of 5,280 feet can be used as standards at lower altitudes. In discussing this problem from the standpoint of the literature on the subject, the conclusion was drawn[1] that between barometric pressures of 610 and 645 mm. of mercury, at which the children were studied, the partial pressure of oxygen was not sufficiently low to influence their basal metabolism. However, in view of the queries raised as to the dependability of the standards of the Child Research Council at other altitudes, further consideration should be given to the question of whether the altitude at which determinations of basal metabolism are made affects the results.

Numerous investigators have studied the effect of altitude on the energy metabolism of human subjects. Observations[2-19] have been made by determining the oxygen consumption of the same individuals first at or near sea level and then at one or more higher elevations. It may be seen from Table I, which gives the percentage changes at different ranges of altitude from the heat production found near sea level, that considerable variation has occurred. However, no marked effect is evident in the case of altitudes below 10,000 feet. The greatest disagreement appears at the higher altitudes and is particularly noticeable in studies made prior to 1913 when the experimental conditions were not always well controlled. Since that time practically all of the observations have been made under basal conditions, and it may be noted that for altitudes between 10,000 and 15,000 feet the deviation from the results obtained at sea level has been considerably less than in the earlier years.

*From the Child Research Council and the Department of Biochemistry, University of Colorado School of Medicine, Denver, and the Oklahoma Agricultural Experiment Station, Stillwater.

This report is taken from the dissertation submitted by Alberta Iliff to the Faculty of the Graduate School of the University of Colorado, June, 1942, in partial fulfillment of the requirements for the degree Doctor of Philosophy.

A preliminary report of this study was made at the meetings of the American Society of Biological Chemists at New Orleans in March, 1940 (A. Iliff, G. M. Kinsman, A. M. Duval and R. C. Lewis, J. Biol. Chem. 133: xlvii, 1940).

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†This author's participation in this study and its publication have been approved by the Oklahoma Agricultural Experiment Station.

TABLE I

PERCENTAGE CHANGES AT DIFFERENT RANGES OF ALTITUDE FROM THE HEAT PRODUCTION ESTABLISHED AT ELEVATIONS BETWEEN SEA LEVEL AND 1,000 FEET

(A SUMMARY OF THE LITERATURE)

	1896-1913				1913-1941			
	No. of reports 7				No. of reports 9			
	Total No. of subjects 15				Total No. of subjects 22			
	RANGE IN ALTITUDE IN FEET ABOVE SEA LEVEL							
	1,000 TO 5,000	5,000 TO 7,500	7,500 TO 10,000	10,000 TO 15,000	1,000 TO 5,000	5,000 TO 7,500	7,500 TO 10,000	10,000 TO 15,000
Percentage changes in energy production	−11	− 7	− 8	− 7	−1	− 8	± 0	−15
	− 7	− 7	− 6	± 0	±0	− 2	+ 2	−11
	− 4	− 3	− 4	+ 3	+1	− 2	+ 4	− 4
	± 0	± 0	+ 5	+ 6		± 0	+ 5	+ 3
	+ 1	± 0	+ 9	+ 6		± 0	+10	+ 3
	+ 2	+ 1	+12	+ 9		+ 1	+16	+ 4
	+ 3	+ 1	+21	+10		+ 2		+ 5
	+ 4	+ 1		+12		+ 2		+ 5
	+ 4	+ 2		+15		+ 2		+ 5
	+ 9	+ 4		+16		+ 3		+ 6
		+ 9		+20		+ 3		+10
		+ 9		+43		+ 3		+10
				+45		+ 4		+11
				+45		+ 5		+12
				+86		+ 5		+13
						+ 5		+13
						+ 8		+14
						+ 8		+16
						+ 9		+17
						+14		+18
								+22
Range of percentage change	−11 to + 9	− 7 to + 9	− 8 to +21	− 7 to +86	−1 to +1	− 8 to +14	± 0 to +16	−15 to +22

Observations made during balloon ascents and in pneumatic chambers have also been used to study the effect of decreased barometric pressure on energy metabolism. The results of such experiments are not pertinent in relation to the problem under consideration, because the factor of acclimatization has entered into only a few of the investigations, and the purpose of the present study has been to determine whether altitude has a significant effect on the basal metabolism of acclimatized individuals.

Although this review of the literature reveals that change of altitude up to 10,000 feet has no significant effect on basal metabolism, the contrary conclusion has been drawn from a few studies[20, 21, 22] made at single elevations. In a later publication[23] the validity of such a conclusion will be questioned. However, inasmuch as these reports have appeared in the literature, the problem has been subjected to further investigation.

Experimental Procedure.—Although the primary purpose of this study was to determine whether the normal values of basal metabolism for children established at the altitude of Denver could be used as standards at lower altitudes, it was impracticable to use children as subjects of investigation. Accordingly, the study was made on seven adults. The basal metabolism of all seven subjects (two men and five women) was measured at Denver, Colo. (altitude, 5,280 feet) and at Stillwater, Okla. (altitude, 910 feet), and four of the sub-

jects (one man and three women) were also studied at Eldora, Colo. (altitude, 8,720 feet). During the period of residence at each place, no subject changed from that altitude by more than 1,000 feet for longer than one hour.

Three of the subjects (L., D., and I.) had been residents of Denver for at least ten years and had not been to an altitude lower than 5,000 feet for three months or longer before the first series of determinations was made at Denver. Between the completion of this initial period and the determinations in Stillwater, Subject L. spent four days at an altitude of about 900 feet but returned to Denver for 5 days just prior to leaving for Oklahoma. Subjects D. and I. remained in Denver until their departure for Stillwater. The other four subjects (N., K., H., and B.) had been residents in the Middle West for at least two years and had not been to an altitude higher than 1,000 feet during the three months before the first determinations were made on them in Stillwater.

After the initial period in Denver (Denver I), the three subjects, L., D., and I., motored to Stillwater on May 15 and 16 and remained there for twenty-four days. During their stay, basal metabolism determinations and blood studies were made on them and on the four residents of Stillwater. On June 10 the three subjects (L., D., and I.) returned to Denver where further determinations were made for a period of several weeks (Denver II). Subject H. came to Denver on July 1, and observations were made on her over a period of eight months while she remained at the higher altitude. On July 23 Subject K. arrived in Denver where determinations were made on her until August 5. These two latter subjects had remained in Oklahoma until their departure for Denver. In December, Subjects N. and B. came to Denver for determinations during a period of eleven days. They had been residing in Illinois at an altitude of about 700 feet since the end of the observations on them in Stillwater. Even though these studies on Subjects N., K., H., and B. constituted the first period of observations on them in Denver, this period has been designated, for the sake of clarity, as Denver II in the report to follow. Thus, for all seven subjects, the period known as Denver II indicates one following a change from a relatively low to a higher altitude.

Subjects D., I., and K. went to Eldora, Colo. on August 5 and were joined there by Subject L. on August 7. Determinations were made on all of them until August 21 when they returned to Denver where further observations were made in a final period at Denver (Denver III).

During the course of this study the living habits of the subjects were not materially changed at the different places. Indeed, the body weight and general physical condition of each subject varied little except for Subject L. who was ill just before the observations at Eldora. This illness occurred during a non-experimental period in Denver, and the weight of this subject remained relatively low throughout the period at Eldora and the final period at Denver.

The determinations of basal metabolism were made by the same individuals with the same closed circuit apparatus, a No. M 84 McKesson Metabolor, at all altitudes. From five to thirteen determinations were made on each subject in the initial periods, whether at Denver or at Stillwater. After each change in altitude, except during the third period in Denver, almost daily determinations were made for ten or more days. The subjects had fasted for at least twelve

hours and had rested on a comfortable bed in the laboratory for forty-five minutes before the first test to measure oxygen consumption was performed. In six instances only two tests were procured, since the variation in oxygen consumption from the mean was no greater than 2 per cent. In all other instances at least three tests of four minutes each or more were obtained. If the oxygen consumption of any one of the three tests showed a deviation of more than 4 per cent from the mean, another test was procured. When more than three tests were made, the deviation from the mean seldom exceeded 5 per cent. The mean value for oxygen consumption was used to calculate the heat production. As the respiratory quotient was not determined, 4.825 was used as the calorific value for each standard liter of oxygen. The basal metabolism calculated by the use of this factor is reported as calories per hour per square meter of body surface. The surface area values were obtained from a nomogram constructed by Wilson and Wilson[24] from the Du Bois and Du Bois height-weight formula.[25]

Eleven alcohol check determinations were made at intervals during the study, and these gave an average value of 0.4 per cent above the theoretical oxygen consumption with a range of from −1.5 to +2.9 per cent.

Blood studies made on each subject appear in a separate report by Lewis, Iliff, Duval, and Kinsman.[26] The values obtained for number of red cells, hemoglobin content, and packed red cell volume show that physiologic adjustment to altitude occurred comparatively early in the period of residence following each change in altitude.

EXPERIMENTAL RESULTS

The results of the individual determinations of basal metabolism are not presented here but may be found elsewhere.* For the most part, the values for each subject showed comparatively little variation in each period. However, it was found that on the first two days for Subjects L. and D. at Stillwater, on the first day for Subject I. at Stillwater, and on the first day for Subject H. at Denver the values for basal heat production (calories per hour per square meter) were markedly greater than those obtained subsequently during that period. Since these six high values were obtained in each case at the beginning of a period following a change in altitude and were distinctly out of line, they have been omitted in the calculation of the averages for the periods in which they occurred. However, even if these high results had been included, the means for these periods would not have been materially changed. Since in three instances the high initial values were observed on subjects who had moved from a higher to a lower altitude and in one case on a subject who had moved in the opposite direction, it is unlikely that the direction of change of altitude is the factor which caused these variations.

Table II shows the mean values for the calories per hour per square meter of body surface for the different periods as well as the standard errors of the means. From the latter values it may be observed that the means are reliable in all cases. Subjects N., I., K., H., and B show a close agreement in the means for the three altitudes. The values for Subject L. averaged 31.7 calories per

*The data of individual basal metabolism tests may be found in Part I, Table 2, of the dissertation of Alberta Iliff, "The Effect of Altitude on Certain Physiological Functions of Human Subjects," which is on deposit in the Charles Denison, M.D., Memorial Library, University of Colorado School of Medicine, Denver. Microfilm copy of the table (pp. 19 to 32, incl.) may be obtained from the Librarian of the University of Colorado, Boulder, Colo.

TABLE II

NUMBER OF TESTS AND MEAN VALUES OF CALORIES PER HOUR PER SQUARE METER ± THE STANDARD ERROR OF THE MEAN FOR EACH SUBJECT AT EACH PERIOD

SUBJECT	DENVER I		STILLWATER		DENVER II		ELDORA		DENVER III	
	NO. OF TESTS	MEAN ± THE STANDARD ERROR, THEREOF	NO. OF TESTS	MEAN ± THE STANDARD ERROR, THEREOF	NO. OF TESTS	MEAN ± THE STANDARD ERROR, THEREOF	NO. OF TESTS	MEAN ± THE STANDARD ERROR, THEREOF	NO. OF TESTS	MEAN ± THE STANDARD ERROR, THEREOF
N. (♂)			8	32.9 ± 0.36	10	33.5 ± 0.36				
L. (♂)	10	31.7 ± 0.27	15	34.4 ± 0.24	10	34.2 ± 0.24	10	34.2 ± 0.20	4	34.0 ± 0.55
D. (♂)	10	34.4 ± 0.45	12	32.0 ± 0.28	17	32.7 ± 0.27	11	33.1 ± 0.45	7	32.5 ± 0.53
I. (♂)	10	31.8 ± 0.39	11	31.8 ± 0.36	13	31.1 ± 0.21	13	31.5 ± 0.21	4	31.5 ± 0.56
K. (♂)			13	31.6 ± 0.42	7	31.4 ± 0.31	13	31.3 ± 0.34	3	30.5 ± 0.01
H. (♂)			5	30.5 ± 0.44	15	30.4 ± 0.33				
B. (♀)			7	31.9 ± 0.21	11	32.4 ± 0.24				

hour per square meter in the initial period in Denver and from 34.0 to 34.4 calories in the four subsequent periods. The values for Subject D. averaged 34.4 calories per hour per square meter in the first period in Denver, but the subsequent means were all lower and varied between 32.0 and 33.1 calories. It should be noted that the changes after the initial period for Subjects L. and D. are in the reverse direction from one another.

TABLE III

SIGNIFICANCE OF THE DIFFERENCE BETWEEN MEANS OF BASAL METABOLISM FOR EACH SUBJECT AT DIFFERENT PERIODS AS INDICATED BY THE RATIO OF THE DIFFERENCE BETWEEN THE TWO MEANS IN EACH CASE TO THE STANDARD ERROR OF THE DIFFERENCE

DATA USED	$\frac{m_1 - m_2}{\sigma_D}$* FOR SUBJECTS						
	N. (♂)	L. (♂)	D. (♀)	I. (♀)	K. (♀)	H. (♀)	B. (♀)
Denver I and Stillwater		7.6†	4.5†	0.0			
Denver II and Stillwater	1.1	0.6	1.8	1.7	0.5	0.3	1.6
Denver III and Stillwater		0.7	0.8	0.5	2.6		
Denver I and Denver II		6.9†	3.2†	1.7			
Denver I and Denver III		3.7†	2.7	0.6			
Denver II and Denver III		0.4	0.4	0.6	2.7		
Denver I and Eldora		7.2†	2.0	0.8			
Denver II and Eldora		0.2	0.7	1.2	0.2		
Denver III and Eldora		0.3	0.8	0.0	2.2		
Stillwater and Eldora		0.9	2.0	0.8	0.7		

*The values used in the calculation of this formula are: m_1 = the mean obtained in one period, m_2 = the mean obtained in the other period, σ_D = the standard error of the difference or $\sqrt{\sigma^2_{m1} + \sigma^2_{m2}}$, where σ_{m1} = the standard error of the mean of the values in one period and σ_{m2} = the standard error of the mean of the values in the other period.

†The values so indicated are significant because the difference in the means is 3.0 or more times the standard error of the difference between the means.

Table III has been constructed to show whether there are any significant differences between the averages for the calories per hour per square meter of body surface obtained in the various experimental periods. The value given in each case is the ratio of the difference between two means to the respective standard error of this difference.[27] It may be seen that the differences between the basal heat production for any two periods are not significant for Subjects N., I., K., H., and B. On the other hand, the values for Subject L. show a significant difference between the initial period at Denver and each of the other periods. Similarly, the values for Subject D. indicate a significant difference between the basal heat production for the first period in Denver and those for the period in Stillwater and for the second period in Denver, respectively. However, when the comparison does not include the first period in Denver, it may be seen that the differences between the means of any other two periods for Subjects L. and D. are not significant. Since similar significant differences to those noted for Subjects L. and D. between the means of the initial period at Denver and those found at other altitudes are shown by these subjects between the means for the first period in Denver and for one or more subsequent periods at this same place, it is evident that factors other than altitude are responsible for the aberrant findings for these subjects in the initial period at Denver. Accordingly, it may be concluded that change in altitude up to 8,720 feet had no effect on the basal metabolism of any of the subjects studied.

In the present study lower values for intraindividual variability were found than those reported by Berkson and Boothby.[28] When the composite data for all periods on men and women, respectively, were treated by the procedure used by these authors on their similar data, the values for one standard deviation were found to be 1.27 calories per hour per square meter of body surface for the men and 1.19 calories for the women. Berkson and Boothby found 1.33 calories for men and 1.61 calories for women. Table IV, which gives the number of tests and the value for one standard deviation from the mean for each subject in each period as well as for the entire study, shows the greatest variability for any one period for the men to be 1.09 calories for Subject N. at Denver and for the women, 1.44 calories for Subject K. at Stillwater and for Subject D. at Eldora. Even when the calculation is made on all determinations of each subject, the highest values found are 1.30 calories on Subject L. for the men and 1.42 calories on Subject D. for the women. All of these values show a relatively low intraindividual variability.

TABLE IV

NUMBER OF TESTS AND VALUES FOR ONE STANDARD DEVIATION (σ)* FROM THE MEAN VALUE FOR CALORIES PER HOUR PER SQUARE METER OF BODY SURFACE OF EACH SUBJECT FOR EACH EXPERIMENTAL PERIOD

PERIOD	N. (♂)		L. (♂)		D. (♀)		I. (♀)		K. (♀)		H. (♀)		B. (♀)	
	NO. OF TESTS	σ	NO. OF TESTS	σ	NO. OF TESTS	σ	NO. OF TESTS	σ	NO. OF TESTS	σ	NO. OF TESTS	σ	NO. OF TESTS	σ
Denver I			10	0.82	10	1.35	10	1.18						
Stillwater	8	0.98	15	0.89	12	0.90	11	1.14	13	1.44	5	0.88	7	0.49
Denver II	10	1.09	10	0.65	17	1.08	13	0.73	7	0.74	15	1.25	11	0.73
Eldora			10	0.61	11	1.44	13	0.66	13	1.19				
Denver III			4	0.95	7	1.30	4	0.86	3	0.09				
All Tests	18	1.08	49	1.30	57	1.42	51	0.97	36	1.21	20	1.17	18	0.69

*The formula used for this calculation was that of Boothby, Berkson and Dunn[29] $\sigma = \sqrt{mS - M^2}$, where mS is the mean of the squares of the differences of the observed values from the mean value of the individual under consideration and M is the mean of these differences.

Although the primary purpose of this investigation was to determine the effect of altitude on basal metabolism, the nature of the study makes possible some observations on geographic location. Talbot, Wilson, and Worcester[30] believe that this is one of the factors which affects the basal heat production, and Talbot and Talbot[31] suggest that the principal reason for the disagreement between the standards of basal metabolism for children established by them in Boston and those reported for the children of the Child Research Council at Denver[1] is the difference in geographic location between the two cities. Boston is 2.8° farther north than Denver, and differences in latitude of this same order are found between the places at which the present study was made. Eldora and Denver are 3.0° and 2.6°, respectively, farther north than Stillwater. However, no evidence has been obtained in the present study which would indicate that differences in latitude of this magnitude cause significant differences in basal heat production. Thus, no confirmation can be given here to support the Talbots' contention that the disagreement between the standards of basal metabolism for children established by them in Boston

and by the studies of the Child Research Council in Denver is due to the difference in geographic location.

Whether the illness of Subject L. in Denver just prior to the experimental period in Eldora had any effect on the results obtained on him at the higher altitude might be questioned. However, the fact that the basal heat production in his case remained unchanged, just as it did in the other Subjects (D., I., and K.) who were also studied at Eldora, indicates that the illness had no significant effect.

In conclusion, it may be well to comment upon whether the question which prompted the present study has been answered; namely, whether the standards of basal metabolism established on children of the Child Research Council at Denver may be used at other altitudes. As has been explained earlier in this paper, practical considerations necessitated the use of adults as subjects in this investigation. However, there appears to be no valid reason for expecting a difference between the effect of altitude on children and on adults. Accordingly, since change of altitude did not affect the basal metabolism of the adults studied, the standards[1, 32] for the basal metabolism of children established at Denver may be considered reliable for use at altitudes below 9,000 feet.

SUMMARY

1. The basal metabolism of seven adults (five women and two men) was determined at Denver, Colo. (altitude, 5,280 feet) and at Stillwater, Okla. (altitude, 910 feet). Four of the subjects (three women and one man) were also studied at Eldora, Colo. (altitude, 8,720 feet).

2. The subjects remained at each altitude sufficiently long to become acclimatized as judged by certain observations made on the blood for the measurement of physiologic adaptation. During the period of adjustment to the changes in altitude, except after the return to Denver from a higher altitude, determinations of basal metabolism were made almost daily and also for some days after acclimatization had been accomplished.

3. Change in altitude within the limits of the elevations studied did not affect the basal metabolism.

4. Changes in geographic location within the limits and under the conditions experienced in this study had no effect on the basal metabolism.

5. A critical review of the literature shows that change in altitude at levels below 9,000 feet does not affect the basal metabolism and this view is confirmed by the present study. Thus, it may be concluded that for elevations below this level the standards[1, 32] for the basal metabolism of children established by the investigations of the Child Research Council at Denver are reliable.

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THE EFFECT OF CHANGE OF ALTITUDE ON THE BLOOD OF HUMAN SUBJECTS*

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IN CONJUNCTION with an investigation to determine the influence of altitude on the basal metabolism of adult human subjects, studies on their blood were conducted primarily as a means of ascertaining when physiologic adjustment to the changes in altitude occurred. These studies consisted of the determination of the number of red cells, the hemoglobin content, the packed red cell volume, and the specific gravity. Although it has often been observed that physiologic alterations in the blood occur with changes in altitude, seldom has such an opportunitiy as the one encountered in this study been afforded to observe the effects of altitude on the same individuals by identical techniques performed by the same workers and with almost no change in the living habits of the subjects.

EXPERIMENTAL PROCEDURE

The subjects of the study were five women and two men, and the altitudes at which the observations were made were 910 feet, 5,280 feet, and 8,720 feet above sea level. Only four of the subjects were studied at the highest altitude. The conditions under which the experiments were conducted are described in detail in our report on the effect of altitude on basal metabolism.[1]

Freely flowing blood was collected from the fingertip directly into small vials in which three drops of 0.5 per cent solution of heparin had been allowed to dry. The blood was usually drawn before breakfast, but occasionally it was taken at other times of day than early in the morning. By making a relatively deep puncture, it was possible to collect readily from six to ten drops (approximately 0.5 c.c.) of blood, and this gave adequate amounts for the determination of the number of red cells, the hemoglobin content, the packed red cell volume, and the specific gravity.

The red cell counts were made on at least two dilutions from each sample of blood. Hayem's solution was used in all cases. The pipettes and the Levy-Hausser counting chambers had been certified by the Bureau of Standards. More than 1,000 cells were counted from each pipette, and the mean value of the number of red cells per cubic millimeter for the two dilutions has been used.

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This report is taken from the dissertation submitted by Alberta Iliff to the Faculty of the Graduate School of the University of Colorado, June, 1942, in partial fulfillment of the requirements for the degree Doctor of Philosophy.

A preliminary report of this study was made at the meetings of the American Society of Biological Chemists at New Orleans in March, 1940 (Kinsman, G. M., A. M. Duval, A. Iliff and R. C. Lewis, J. Biol. Chem. **133**: liii, 1940).

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†This author's participation in this study and its publication have been approved by the Oklahoma Agricultural Experiment Station.

The hemoglobin content was determined by the acid hematin method. The Klett hemoglobinometer used for all readings was calibrated by comparison with the Van Slyke-Neill[2] manometer method of gas analysis for the determination of the oxygen-carrying capacity of the hemoglobin. In order to assure a constancy of light, the same daylight lamp was used at all altitudes, and a distance of 12 cm. from the light to the tubes was always maintained. Immediately after introducing the blood into the 0.1 N HCl, the solution was shaken for 3.75 minutes, and the readings were made within the following two minutes. This procedure had been found to give values very close to those obtained by the Van Slyke-Neill method when the conversion factor of 1.34 c.c. of oxygen for 1 Gm. of hemoglobin was employed. Two determinations that checked within 2 per cent were obtained on each sample of blood. The mean of these values has been used as the hemoglobin content of the blood.

The volume of packed red cells was determined by the use of Van Allen[3] hematocrit tubes. Instead of the clips or rubber bands customarily used to seal the lower end of the capillary tube, melted beeswax was employed for this purpose. The introduction of blood into the capillary tube was accomplished by drawing the blood up to the 100 per cent mark. After wiping the excess blood from the tip with a gauze sponge, the column of blood was carefully drawn up the capillary tube about ½ cm. Melted beeswax was then applied to the lower end of the tube with a camel hair brush and allowed to harden for three minutes. The tube was centrifuged at 2,500 revolutions per minute until there was no further change in the volume of the packed red cells. After centrifugation, the total volume of blood read at or very slightly below the 100 per cent mark, which showed that the air introduced into the lower end of the tube had escaped completely and that no blood had been lost. Triplicates from each sample of blood were run and usually the readings for packed red cell volume in all three tubes showed agreement. Unless two of the readings were within 2 per cent of one another, the determinations were repeated. The mean value for the two or three acceptable determinations has been used.

The specific gravity was determined by the use of the falling drop apparatus of Barbour and Hamilton,[4, 5] which was standardized by the use of solutions of potassium sulfate with specific gravities of 1.0551, 1.0645 and 1.0743. In the determination of the specific gravity, triplicate readings of the rate of fall of blood and of standard, respectively, were considered acceptable when they agreed within 3 per cent. The means of these values for the blood and for the standard were used in the calculation of the specific gravity of the blood.

In addition to the blood studies, measurements of pulse rate and blood pressure were made several times on each subject at each altitude. Since the results were normal in all cases and no significant variation occurred on changing altitude, the data will not be reported.

RESULTS AND DISCUSSION

Table I gives the results of determinations of number of red cells, hemoglobin content, packed red cell volume, and specific gravity on bloods collected in Denver at different times of day on Subjects D., I., and L. at a time when they were thoroughly acclimatized. It shows that in none of these determinations

TABLE I

COMPARISON OF THE DETERMINATIONS OF THE NUMBER OF RED CELLS, THE AMOUNT OF HEMOGLOBIN, THE PACKED RED CELL VOLUME, AND THE SPECIFIC GRAVITY OF THE BLOOD OBTAINED ON THE SAME DAY UNDER FASTING AND NONFASTING CONDITIONS

SUBJECT	RED CELLS (MILLIONS PER CU. MM.)		HEMOGLOBIN (GM. PER 100 ML.)		CELL VOLUME (VOLUMES PER CENT)		SPECIFIC GRAVITY	
	FASTING	NON-FASTING	FASTING	NON-FASTING	FASTING	NON-FASTING	FASTING	NON-FASTING
D. (♀)	5.30	5.26	14.6	14.4	45.4	43.0	1.0620	1.0571
	5.24	5.16	14.5	14.4	46.0	46.1	1.0605	1.0602
	4.99	4.96	14.4	13.9	44.6	43.9	1.0627	1.0616
I. (♀)	5.32	5.26	14.9	15.0	46.0	45.9	1.0608	1.0663
	5.32	5.33	15.8	15.0	46.1	45.4	1.0627	1.0618
	5.03	5.01	15.0	14.9	45.0	43.1	1.0594	1.0631
	5.35	5.27	15.6	15.0	47.3	45.3	1.0620	1.0623
L. (♂)	6.08	6.28	17.0	17.1	50.6	51.3	1.0671	1.0661
	6.30	6.33	17.1	16.6	48.8	48.8	1.0675	1.0673
	6.26	6.14	17.0	16.2	50.4	50.9	1.0673	1.0669

is there a significant difference between the values obtained on fasting morning bloods and on those collected later during the day. Consequently, the few determinations that were made on blood collected under other than fasting conditions have been included in the data used.

Tables II, III, and IV give the means for the number of red cells, the hemoglobin content and the packed red cell volume, respectively, for the samples of blood taken at each altitude from each subject. In calculating these means, all values have been used except those obtained on the first three days after a change in altitude. The three-day interval was chosen arbitrarily, since the

TABLE II

THE MEAN, THE STANDARD ERROR AND THE STANDARD DEVIATION OF THE MEAN, AND THE COEFFICIENT OF VARIATION FOR THE NUMBER OF RED CELLS OF THE BLOOD. (FIRST THREE OF THESE VALUES EXPRESSED AS MILLIONS OF RED CELLS PER CUBIC MILLIMETER OF BLOOD)

		SUBJECTS						
		N. (♂)	L. (♂)	D. (♀)	I. (♀)	K. (♀)	H. (♀)	B. (♀)
Stillwater (Altitude, 910 feet)	No. of tests	4	7	9	10	6	4	3
	Mean	5.38	5.56	4.62	4.82	4.47	4.72	4.55
	Standard error	0.064	0.026	0.034	0.081	0.069	0.100	0.029
	Standard deviation	0.112	0.065	0.098	0.244	0.153	0.172	0.041
	Coefficient of variation (per cent)	2.1	1.2	2.1	5.1	3.4	3.6	0.9
Denver (Altitude, 5,280 feet)	No. of tests	6	8	20	19	7	12	6
	Mean	5.84	6.30	4.79	5.08	4.83	4.64	4.87
	Standard error	0.113	0.092	0.044	0.032	0.062	0.083	0.037
	Standard deviation	0.253	0.244	0.194	0.137	0.153	0.274	0.083
	Coefficient of variation (per cent)	4.3	3.9	4.0	2.7	3.2	5.9	1.7
Eldora (Altitude, 8,720 feet)	No. of tests		7	9	9	9		
	Mean		5.99	4.93	5.35	4.82		
	Standard error		0.063	0.028	0.040	0.032		
	Standard deviation		0.154	0.080	0.114	0.091		
	Coefficient of variation (per cent)		2.6	1.6	2.1	1.9		

TABLE III

THE MEAN, THE STANDARD ERROR AND THE STANDARD DEVIATION OF THE MEAN, AND THE COEFFICIENT OF VARIATION FOR THE HEMOGLOBIN CONTENT OF THE BLOOD (FIRST THREE OF THESE VALUES EXPRESSED AS GRAMS OF HEMOGLOBIN PER 100 MILLILITERS OF BLOOD)

		SUBJECTS						
		N. (♂)	L. (♂)	D. (♀)	I. (♀)	K. (♀)	H. (♀)	B. (♀)
Stillwater (Altitude, 910 feet)	No. of tests	4	7	10	10	7	4	3
	Mean	14.05	15.70	13.44	13.85	12.86	12.75	12.33
	Standard error	0.098	0.210	0.086	0.189	0.270	0.459	0.249
	Standard deviation	0.170	0.516	0.258	0.568	0.662	0.795	0.352
	Coefficient of variation (per cent)	1.2	3.3	1.9	4.1	5.1	6.2	2.8
Denver (Altitude, 5,280 feet)	No. of tests	6	8	20	20	7	12	6
	Mean	16.28	16.78	14.10	15.18	14.13	12.92	13.00
	Standard error	0.086	0.147	0.110	0.077	0.174	0.158	0.188
	Standard deviation	0.192	0.390	0.478	0.336	0.425	0.524	0.420
	Coefficient of variation (per cent)	1.2	2.3	3.4	2.2	3.0	4.0	3.2
Eldora (Altitude, 8,720 feet)	No. of tests		7	9	9	9		
	Mean		16.73	14.83	16.06	14.91		
	Standard error		0.171	0.212	0.274	0.289		
	Standard deviation		0.419	0.599	0.577	0.818		
	Coefficient of variation (per cent)		2.5	4.0	3.6	5.5		

TABLE IV

THE MEAN, THE STANDARD ERROR AND THE STANDARD DEVIATION OF THE MEAN, AND THE COEFFICIENT OF VARIATION FOR THE PACKED RED CELL VOLUME OF THE BLOOD (FIRST THREE OF THESE VALUES EXPRESSED AS VOLUMES PER CENT OF BLOOD)

		SUBJECTS						
		N. (♂)	L. (♂)	D. (♀)	I. (♀)	K. (♀)	H. (♀)	B. (♀)
Stillwater (Altitude, 910 feet)	No. of tests	4	6	10	10	7	4	3
	Mean	45.52	47.70	41.98	41.29	39.59	39.20	38.43
	Standard error	0.655	0.276	0.355	0.447	1.058	0.641	1.761
	Standard deviation	1.134	0.462	1.065	1.341	2.590	1.111	2.490
	Coefficient of variation (per cent)	2.5	1.0	2.5	3.2	6.5	2.8	6.5
Denver (Altitude, 5,280 feet)	No. of tests	6	8	20	18	7	12	6
	Mean	47.02	50.64	43.32	45.58	41.20	39.58	39.33
	Standard error	0.316	0.459	0.273	0.366	0.470	0.443	0.631
	Standard deviation	0.707	1.215	1.192	1.508	1.152	1.470	1.410
	Coefficient of variation (per cent)	1.5	2.4	2.8	3.3	2.8	3.7	3.6
Eldora (Altitude, 8,720 feet)	No. of tests		7	9	9	9		
	Mean		49.09	45.62	48.53	41.24		
	Standard error		0.661	0.812	0.516	0.710		
	Standard deviation		1.619	2.296	1.458	2.009		
	Coefficient of variation (per cent)		3.3	5.0	3.0	4.9		

changes observed in the different subjects showed no constancy with respect to the time of the beginning of the change or to its degree. In addition to the mean in each case, the tables give the standard error of the mean, the standard deviation from the mean, and the coefficient of variation. These statistical values show that there is relatively little scatter in the data obtained at any altitude for any of the subjects. When the means for each subject at the different altitudes are compared, it is seen that with an increase in altitude there were increases in eight instances for the number of red cells, in ten instances for the hemoglobin content, and in nine instances for the packed red cell volume out of eleven possibilities in each case. Thus, in the majority of instances adjustment to a higher altitude was manifested by increases in all three of these values.

Exceptions to the general rule that increases in the number of red cells, in the hemoglobin content, and in the packed red cell volume occur with an increase in altitude are to be found in some instances in the cases of Subjects H., K., and L. Practically no difference in the means occurred in any of these values when Subject H. moved from Stillwater (altitude, 910 feet) to Denver (altitude, 5,280 feet) or in the number of red cells or in the packed red cell volume when Subject K. went from Denver to Eldora (altitude, 8,720 feet). No reasons are evident for the inconsistencies found in these cases. When the mean values for Subject L. during the period at Eldora are compared with those obtained during the experimental periods in Denver, definite decreases in number of red cells and in packed red cell volume and practically no change in hemoglobin content are observed. An illness experienced by this subject during a nonexperimental period in Denver just prior to his departure for Eldora may

TABLE V

THE MEAN, THE STANDARD ERROR AND THE STANDARD DEVIATION OF THE MEAN, AND THE COEFFICIENT OF VARIATION FOR THE SPECIFIC GRAVITY OF THE BLOOD (FIRST THREE OF THESE VALUES EXPRESSED IN TERMS OF SPECIFIC GRAVITY)

		SUBJECTS						
		N. (♂)	L. (♂)	D. (♀)	I. (♀)	K. (♀)	H. (♀)	B. (♀)
Stillwater (Altitude, 910 feet)	No. of tests	4	7	10	10	7	4	3
	Mean	1.0646	1.0660	1.0614	1.0610	1.0581	1.0594	1.0572
	Standard error	0.0024	0.0039	0.0009	0.0012	0.0040	0.0016	0.0007
	Standard deviation	0.0042	0.0096	0.0028	0.0035	0.0099	0.0028	0.0010
	Coefficient of variation (per cent)	0.4	0.9	0.3	0.3	0.9	0.3	0.1
Denver (Altitude, 5,280 feet)	No. of tests	6	8	19	16	7	12	6
	Mean	1.0656	1.0672	1.0611	1.0624	1.0607	1.0580	1.0580
	Standard error	0.0015	0.0034	0.0009	0.0008	0.0013	0.0005	0.0008
	Standard deviation	0.0033	0.0090	0.0039	0.0033	0.0032	0.0017	0.0017
	Coefficient of variation (per cent)	0.3	0.8	0.4	0.3	0.3	0.2	0.2
Eldora (Altitude, 8,720 feet)	No. of tests		7	9	9	9		
	Mean		1.0667	1.0629	1.0650	1.0633		
	Standard error		0.0032	0.0008	0.0013	0.0014		
	Standard deviation		0.0079	0.0022	0.0036	0.0040		
	Coefficient of variation (per cent)		0.7	0.2	0.3	0.4		

account for his failure to have attained higher means for the number of red cells, hemoglobin content, and packed red cell volume at the higher altitude. Four days before he went to the higher altitude, determinations on his blood showed that the number of red cells had fallen to 5.14 millions per cubic millimeter and that the hemoglobin content had dropped to 15.8 Gm. per 100 ml. of blood. Although recovery was rapid, as shown by the fact that six days later or two days after reaching the higher altitude the number of red cells had increased to 5.75 millions per cubic millimeter and the hemoglobin to 17.2 Gm. per 100 ml. of blood, the mean for these values and for packed red cell volume during the period at Eldora probably did not reach the height they would have attained, had this period not followed so closely upon his illness.

Table V gives the mean, the standard error and the standard deviation of the mean, and the coefficient of variation for the specific gravity of the blood at the different altitudes. For any given altitude very little intervariation or intravariation occurs. However, when one compares the mean of each subject at one altitude with that of the next higher altitude, it may be seen that in eight out of eleven possibilities slight but definite increases in specific gravity take place. Since the specific gravity of red blood cells is approximately 1.090 and that of serum 1.027,[6] each 5 per cent change in packed red cell volume would be expected to result in a parallel change of 0.003 in the specific gravity of the

TABLE VI

SIGNIFICANCE OF THE DIFFERENCE BETWEEN THE MEANS OBTAINED AT A HIGHER AND A LOWER ALTITUDE, RESPECTIVELY, FOR NUMBER OF RED CELLS, HEMOGLOBIN CONTENT, PACKED CELL VOLUME, AND SPECIFIC GRAVITY AS INDICATED BY THE RATIO OF THE DIFFERENCE BETWEEN THE TWO MEANS IN EACH CASE TO THE STANDARD ERROR OF THE DIFFERENCE

DIFFERENCES IN MEANS		$\frac{M_1 - M_2}{\sigma_D}$* FOR SUBJECTS						
FOR	OBTAINED AT	N. (♂)	L. (♂)	D. (♀)	I. (♀)	K. (♀)	H. (♀)	B. (♀)
Number of red cells	Denver and Stillwater	3.6†	7.7†	3.0†	3.0†	4.0†	−0.6	6.9†
	Eldora and Denver		−2.7	2.6	5.1†	−0.2		
	Eldora and Stillwater		6.4†	6.8†	5.8†	4.6†		
Hemoglobin content	Denver and Stillwater	17.2†	4.2†	4.7†	6.5†	4.0†	0.3	2.1
	Eldora and Denver		−0.2	3.1†	3.1†	2.3		
	Eldora and Stillwater		3.8†	6.1†	6.6†	5.2†		
Cell volume	Denver and Stillwater	2.0	5.5†	3.0†	7.4†	1.4	0.5	0.5
	Eldora and Denver		−1.9	2.7	4.7†	0.1		
	Eldora and Stillwater		1.9	4.1†	10.6†	1.3		
Specific gravity	Denver and Stillwater	0.4	0.2	−0.2	1.4	0.6	−0.9	0.7
	Eldora and Denver		−0.1	1.0	1.5	1.3		
	Eldora and Stillwater		0.1	1.1	2.4	1.1		

*The values used in the calculation of this formula are: m_1 = the mean obtained at the higher altitude, m_2 = the mean obtained at the lower altitude, σ_D = the standard error of the difference or $\sqrt{\sigma^2_{m1} + \sigma^2_{m2}}$, where σ_{m1} = the standard error of the mean of the values at the higher altitude and σ_{m2} = the standard error of the mean of the values at the lower altitude.

†The values so indicated are significant because the difference in the means is 3.0 or more times the standard error of the difference between the means.

whole blood. It is interesting to note that, except for Subjects D. and H. in changing from Stillwater to Denver and for Subject K. in going from Denver to Eldora, the alterations in specific gravity which do occur are in the direction and of the approximate magnitude to be expected from the changes in packed red cell volume shown in Table IV.

Table VI has been constructed to show whether the changes in the number of red cells, in the amount of hemoglobin, in the packed red cell volume, and in the specific gravity of the blood, which have been observed to occur with an increase in altitude, are significant. In eleven out of fifteen possibilities significant increases in the number of red cells and in the amount of hemoglobin occurred with an increase in altitude. Fewer marked changes were observed in packed red cell volume, for only three of the seven subjects showed significant increases with a change in residence from Stillwater to Denver, and only one of these showed a significant increase in changing from Denver to Eldora. No significant changes in specific gravity were found.

SUMMARY

1. Determinations of the number of red cells, the hemoglobin content, the packed red cell volume, and the specific gravity were made on the blood of seven adult subjects (five women and two men) at Stillwater, Okla. (altitude, 910 feet) and at Denver, Colo. (altitude, 5,280 feet). These same determinations were also made on the blood of one of the men and three of the women at Eldora, Colo. (altitude, 8,720 feet).

2. Although there was considerable variation in the amount of response of different individuals, and in a number of cases no significant change occurred, increases in the number of red cells, in the amount of hemoglobin, and in the packed red cell volume were found in the majority of instances with increases in altitude.

3. Definite but insignificant increases in specific gravity were found to occur in the majority of the cases with each increase in altitude. With few exceptions, these changes in specific gravity were in the direction and of the approximate magnitude to be expected from the alterations in packed red cell volume.

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LABORATORY METHODS

GENERAL

AN ELECTRONIC DEVICE FOR THE LOCATION OF METALLIC FRAGMENTS IN VIVO*

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MOORHEAD has called attention to a foreign body finder.[1] I wish to call attention to a simple electronic device consisting of a minimum of parts which can be used in a similar manner. It is self-contained in a portable case, measuring 6 by 6 by 6 inches and weighing 8 pounds. The circuit to be described may be so adjusted that it has discriminatory powers which may be of use in applications other than the localization of in vivo foreign bodies.

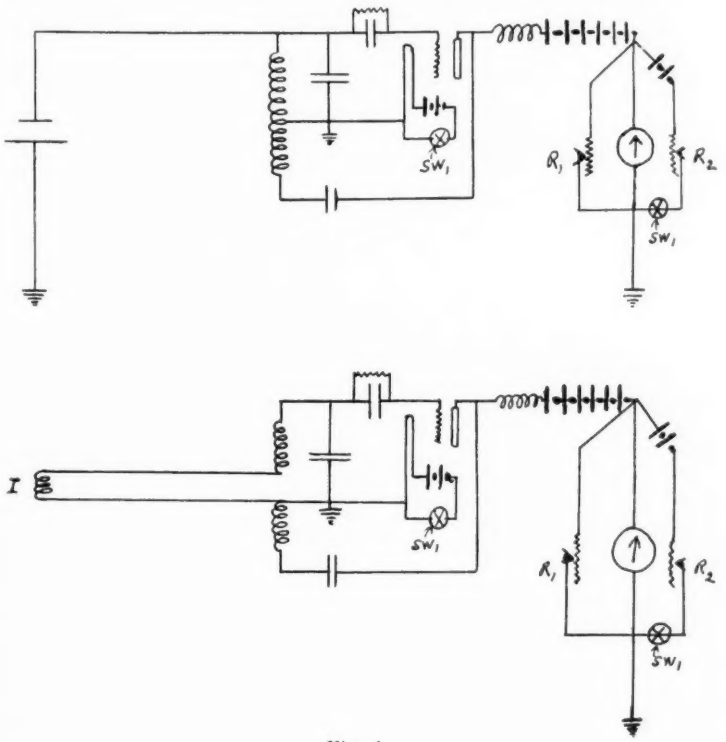

Fig. 1.

The device in question is based upon a modification of an electronic circuit previously described.[2] The schematic circuits here shown (Fig. 1) illustrate the

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modification employed in order to convert the circuit to its present adaptation. From examination of the two circuits it is obvious that the insertion of inductance I with the elimination of the capacity pick-up plate represents the principal change.

Inductance I is incorporated in the circuit by way of a shielded conductor cable one yard in length. It is a small solenoid affixed to the tip of a locator probe 12 inches in length, having a diameter of 0.8 cm., and in the present model is constructed of fiber rod provided with a sterilizable glass covering. The circuit constants are so adjusted that when the locator tip is brought into the presence or in contact with metallic objects characteristic directional deflections occur on the part of the indicating member of the milliammeter placed in the plate circuit.

Table I shows the discriminatory direction deflections obtained when contact is made with the materials listed. Of all the metals listed, iron or alloys of iron gave positive deflections, whereas the remainder of the metals gave negative deflections. There is evidence that for equivalent surface areas soft iron has a greater deflection factor than steel. Similar trends are apparent for metals which cause negative deflections. On the other hand, if discrimination is not desired, the circuit constants may be so adjusted to provide positive or negative deflection for all metals. Experimental evidence indicates that it is possible to "peak" the circuit in such a manner that maximum deflection may be obtained for any one of the series of metals listed.

TABLE I

DIRECTION OF DELFECTION (POSITIVE OR NEGATIVE)

Metals:	
Aluminum	−
Antimony	−
Brass	−
Chromium	−
Cobalt	−
Copper	−
Gold	−
Iron, soft	+
Lead	−
Magnesium	−
Mercury	−
Nickel	−
Silver	−
Sodium	−
Steel	+
Tin	−
Zinc	−
Nonmetals:	
Cement	+
Fiber	+
Glass	+
Mica	+
Paper	+
Wood	+
Solutions:	
Alcohol, absolute	+
Benzol	+
Chloroform	+
Ether	+
Kerosene	+
Water, distilled	+

A check of the maximum air distance between the tip of the locator and the objective for the recognition by demonstrable initial deflection was found to average 1 cm.; however, there existed some differences in this respect with each metal tested. The maximum air distance should be considered in a relative sense in view of the enhanced distance when using a larger probing inductance, and in view of the possibility of substituting a more sensitive meter in the plate circuit. On the other hand, a smaller probe possesses attributes not possessed by larger probes by virtue of its smaller size.

The surfaces of nonmetallic objects were tested, and the maximum air distance for a recognizable change was found to be very small—1 mm. or less—and in most instances contact was essential. In all objects or liquids tested a slight positive deflection occurred. As with the metals the deflection factor varied somewhat with each solution. When metallic objects were tested under water or other liquids, the directional changes noted on deflection were the same as those observed in air. The maximum water distance for recognizable initial change was at least as great as or greater than that found in air.

Experimental implantation of metallic iron and copper test objects (measuring 2 cm. by 2 cm. by 0.5 mm., 1 cm. by 1 cm. by 0.5 mm., 0.5 cm. by 0.5 cm. by 0.5 mm., and 0.25 cm. by 0.25 cm. by 0.5 mm.) was made in the dog. All implants of the above sizes were readily located beneath the skin—skin hair on or off, wet or dry. Deeper burial in the tissues beneath the surface of the skin were also readily located, provided the locator tip was within a distance of 1 cm. or less of the object. Similar implants placed in the abdominal cavity were also readily located.

Actual operation as a foreign body locator is as follows: Sw_1 is turned on. R_1 is rotated to introduce additional shunt resistance in parallel with the meter. The deflection of the meter is controlled to desired scale point by rotation of R_2. Locator is applied to region involved. On contact a small positive deflection occurs. The locator is then moved from one point to another, and any change in deflection, positive or negative, is noted. When such is manifested, the locator tip is manipulated in direction and depth in order to secure the maximum sustained deflection. When such is obtained, the tip of the locator is very near or in contact with the foreign metallic body. Maximum deflection results when the tip surface is parallel to the surface of the object and the minimum deflection results when the tip surface is at right angles to the surface of the object.

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USE OF THE PHOTRONREFLECTOMETER FOR DETERMINATION OF THE OPTIMAL RATIO BETWEEN PNEUMOCOCCUS TYPE I SSS AND ANTIPNEUMOCOCCUS TYPE I RABBIT SERUM

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INTRODUCTION

THE optimal proportions method of precipitin titration, introduced by Dean and Webb[1] in 1926, was based upon their observation that in a series of mixtures containing constant amounts of antiserum and varying amounts of antigen, the fastest precipitation occurred in that mixture which contained antigen and antibody in optimal proportions. Since this technique requires visual comparison of the relative reaction speeds in a number of tubes, its usefulness as a quantitative method of precipitin titration is limited. Consequently, more accurate evaluation of the quantitative relationships between antigen and antibody in the precipitin reaction[2] has resulted from the use of the objective quantitative chemical method of precipitate analysis described by Heidelberger and Kendall.[3]

Although the optimal proportions principle has been challenged on a theoretical basis by Heidelberger and Kabat,[4] the Dean-Webb procedure offers the only available means of determining separate antibody titers in sera containing more than one antibody when tested with solutions containing more than one antigen. Titration of individual antibodies in such a system is of great importance in the study of cross reactions, particularly in determining the proper absorbing dose of antigen. In absorbing an antiserum containing one or more antibodies with a solution containing multiple antigens, exhaustion of the homologous precipitins can be attained only by leaving a supernatant containing different concentrations of each antigen to interfere with subsequent tests for residue antibodies.

It was felt that the problem of precipitin titration in complex systems was of sufficient importance to justify the reinvestigation of the validity of the Dean-Webb optimal ratio. This paper describes a photoelectric method for recording the rate of precipitation and shows that the optimal proportions principle can be used for accurate precipitin titration. The application of the method to the titration of antibodies in complex antigen-antibody systems will be presented in a later paper. Since the photoelectric method has never before been applied to the problem of measuring precipitation rates in antigen-antibody mixtures, the technique is described in detail.

LITERATURE

Ramon[5] was the first to show that the antitoxin titer of a serum could be estimated by observing the first tube to flocculate in a series of tubes containing

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constant volumes of toxin and varying amounts of antitoxin. Dean and Webb[1] reversed the procedure, using a constant volume of serum and varying quantities of antigen. Marrack[6] has referred to these two methods as the alpha (constant antibody) and the beta (constant antigen) procedures. Brown[7] compared the two methods and introduced a third gamma procedure which was carried out after flocculation was complete in the beta series. The tubes were inverted twice, resulting in dispersion of the floccules in some of the mixtures. Brown concluded that there were three types of antigen-antibody reactions, which she described as follows: A type of reaction illustrated by the diphtheria toxin-antitoxin reaction, in which the alpha and beta flocculation zones coincided; that illustrated by the polysaccharide-antibody reaction, in which the alpha and beta methods yielded different flocculation zones but could be made to coincide by using the gamma technique; and that illustrated by the reaction between certain fractions of human serum and homologous antisera, in which the alpha and beta titrations differed and could not be made to agree by the gamma method.

The argument concerning the relationship of the alpha and beta optimal zones may have resulted from differences in opinion concerning the interpretation of the word "floccule." There is no logical basis for reasoning that the fastest reacting mixture in a constant antibody series should necessarily be the fastest reacting mixture in a constant antigen series. Flocculation, however, is a different phenomenon and is evidently related to the proportions of antigen and antibody in the mixture. This is suggested by Brown's[7] work showing that the floccules in certain mixtures could be dispersed after equilibrium had been established, bringing into agreement the different values obtained by the Ramon and Dean-Webb methods in the polysaccharide precipitin system.

The difficulties, inherent in the interpretation of results by either method, are illustrated by the work of Taylor,[8] who reported in 1931 that an optimal Dean-Webb ratio could be obtained for the horse serum antigen-antibody reaction, but that there was no optimal zone in the same system when titered by the Ramon technique. Increasing concentrations of antiserum caused only a speeding up of the reaction without any evidence of inhibition by excess antibody. In 1933, using horse serum and crystalline egg albumin as antigens, he[9] reversed his previous position and stated that the reaction was inhibited by antibody excess.

Although Dean and Webb stated that the optimal mixture was the fastest reacting mixture in a constant antibody-varying antigen series, the reactions in the tubes were described as proceeding from opalescence to turbidity, particulation, and finally flocculation. Since opalescence often appeared in several tubes simultaneously, they suggested that the optimal tube was detected easiest if the reaction were read shortly after particulation occurred, preferably at the time the particles were increasing in size. However, after equilibrium has been established in a constant antibody series, the greatest weight[1] and largest volume[10] of precipitate are found in mixtures containing excess antigen and not in the optimal reacting mixture. This emphasizes the fact that as the reaction progresses, there is a shift in particle size toward the region of antigen excess, suggesting that the mixtures should be compared as early as possible in the reaction.

Theoretical objections to acceptance of the Dean-Webb optimal proportions principle have been expressed by Heidelberger and Kabat[4] as follows:

". . . in a precipitation or agglutination reaction the factors influencing the velocity are imperfectly understood; (2) the position of the flocculation optimum is not independent of the dilution and varies whether the antigen or antibody be diluted; (3) in neither case is the optimum at the point of antibody exhaustion, so that the method gives no information as to the actual agglutinin or precipitin content of a serum."

Although the photoelectric method of measuring precipitation rates does not yield information concerning the actual speed of antigen-antibody union, differences in rate of formation of the light-reflecting precipitated particles can be determined and compared. Objections (2) and (3) are valid, since they refer to end points based on flocculation time and are subject to the differences in interpretation discussed above.

Certain technical difficulties are involved in preparing a series of mixtures for titration by either the Dean-Webb or Ramon method. For accurate visual comparison of the optimal and adjacent tubes, the mixtures should be made as nearly simultaneously as possible. As the ideal of simultaneous mixing is approached, accuracy in pipetting and mixing of the reagents must decrease. Conversely, precise measurement and thorough mixing cause a widening of the time interval between preparation of the various tubes in either series, creating difficulty in making satisfactory comparisons. Smith[11] discussed the problem in detail and suggested that the occurrence of double zones might be caused by improper mixing.

The method presented here avoids these difficulties, since each mixture is made separately and the speed of each reaction, measured by photoelectric means, is recorded permanently and is available for comparison at any time.

MATERIALS AND METHODS

Materials.—Pneumococcus type I polysaccharide was used in all experiments. The preliminary observations were made with a sample of dried polysaccharide furnished by Dr. A. F. Goebel and Dr. T. M. Rivers of the Rockefeller Institute for Medical Research. Another batch of material was obtained from the Lederle Laboratories through the courtesy of Dr. A. L. Joyner and Miss Frances L. Clapp. The polysaccharide was dissolved in sufficient 0.85 per cent saline (containing merthiolate 1:10,000) to make a concentration of 1 mg. per milliliter of solution.

The antiserum in all experiments was refined antipneumococcus type I rabbit serum furnished by the Lederle Laboratories. The antibody content of this serum, as determined in these laboratories, was 5.7 mg. antibody N per milliliter.

Apparatus.—Libby's[12] photronreflectometer was chosen because its usefulness in the precipitin reaction had been demonstrated by Libby[13, 14] and Bukantz, Cooper, and Bullowa[15] who showed that the pneumococcus polysaccharide content of a solution could be determined by observing the galvanometer deflection at a constant time interval after mixing. This instrument is constructed with a small black circular disk in the center of the photoelectric cell directly opposite the circular window of the chamber containing the mixture. Consequently, the

galvanometer reading is dependent upon the number of light rays deflected by particles in the chamber impinging on the periphery of the photoelectric cell.

Preliminary observations showed a short but definite lag in galvanometer response when a stable vaccine suspension was introduced into the apparatus. Before each experiment the galvanometer was adjusted to give a reading of 10 on the scale. Introduction of the clean empty chamber always caused a shift to a value between 2 and 3. The deflection of the empty chamber was determined before each experiment, and this value was subtracted from all readings made during the experiment.

Accuracy.—Sixteen determinations were made with a sample of diluted typhoid vaccine. Four different chambers were used, washed between each determination, and the readings were corrected for the deflection of the empty chamber. The highest deflection was 39.9, the lowest 38.3, the mean 39.0, and the standard deviation 0.40. Since the precipitation reaction involves two reagents and introduces errors in measurement, mixing, and timing, 10 separate experiments were done, using the same antigen and antibody solutions. Readings made five minutes after mixing showed a maximum of 56.6, a minimum 52.8, mean 54.0, and standard deviation 1.34.

Temperature Controls.—All experiments were done at room temperature (21° to 22° C.), the serum and antigen dilutions being kept in a room temperature water bath until used. However, it was observed that the temperature of the fluid in the chamber rose as much as 6° C. in five minutes if the apparatus had been used for seven or eight consecutive experiments. This was controlled partially by continuous passage of a stream of air through a tube inserted in one of the ventilating holes of the apparatus and disconnecting the machine from the electric current for a five-minute interval between experiments. The temperature rise was not eliminated but could be reduced to an almost constant value of 2.1° C. for each five-minute experiment.

Method of Mixing.—Mixing of antigen and antibody was accomplished by expelling 1.4 ml. of the antigen solution into a Wassermann tube containing 1.4 ml. of diluted serum. The mixture was drawn back into the pipette and expelled twice. The third filling of the pipette was transferred to the chamber previously placed in the apparatus.

Reading Times.—An assistant started a stop watch at the time the antigen solution was expelled into the serum. Galvanometer readings were made at ten-second intervals for the first minute, at fifteen-second intervals during the second minute, and at thirty-second intervals for the remaining three minutes. Except for the preliminary observations, all experiments were terminated five minutes after mixing.

Supernatant Examination.—After each experiment the chamber contents were poured into a Wassermann tube and placed in the icebox. After centrifugation the next morning, each supernatant was tested with three dilutions of antibody and three dilutions of antigen.

Calculations.—Each deflection was corrected by subtracting from it the deflection of the empty chamber. A second correction was made for the deflection caused by the control serum. Because of slight bubble formation, the introduction of control serum-saline mixtures caused an increase in deflection followed

by a slow drift back to a stabilized value in thirty to forty seconds. The control values at the appropriate time intervals were subtracted from all readings made during each antigen-antibody experiment.

EXPERIMENTAL

Effect of Flocculation on Galvanometer Deflection.—Since flocculation is followed by a settling out of the precipitate in the chamber, this experiment was planned to determine the effect of prolonged incubation on the galvanometer deflection. The data, obtained before the temperature control experiments were done, were not as accurate as those obtained later, but the results illustrated the limitations of the method after flocculation had occurred.

The mixtures were made in the usual way, but after recording the deflections for the usual five-minute periods, the contents of each chamber were poured into Wassermann tubes. At various intervals over a two-and-a-half-hour period, each mixture was shaken, placed in the chamber, and the deflection recorded.

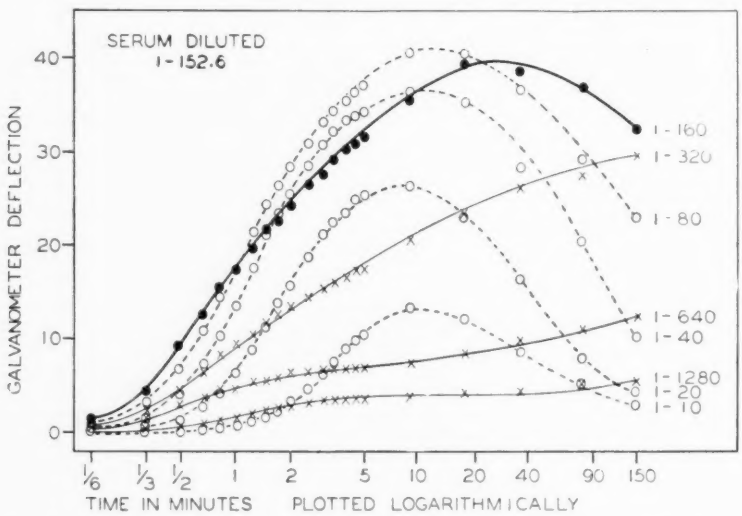

Fig. 1.—Deflection time curves obtained with constant amounts of antibody and various concentrations of antigen over a period of two and one-half hours. Open circles indicate excess antigen was found in the supernatant. Crosses indicate that excess antibody was found in the supernatant. Closed circles indicate that neither antigen nor antibody was found in the supernatant.

The results, plotted in Fig. 1, showed that if observations were continued over a long period of time there was a gradual falling off in galvanometer deflection in the optimal mixture and in mixtures containing excess antigen. Since settling of the precipitate was excluded by shaking of the mixture before placing in the chamber, the fall in deflection resulted from a decrease in the amount of light reflected from the precipitate due either to a reduction in surface-volume ratio or to the absorption of light rays by these very dense suspensions. The precipitates in these mixtures were large and flocculant in contrast to the finely divided turbid suspensions found in all mixtures containing excess of antibody. Although the falling deflection indicated floccule formation, there was no sharp end point and flocculation time could not be estimated. Since none of the mix-

tures showed a decrease in galvanometer deflection during the first five minutes of observation, all subsequent experiments were terminated at this time interval after mixing.

Optimal Proportions in Various Antigen and Antibody Concentrations.—Each of 11 serum dilutions, varying from 1:6.25 to 1:200, was tested with nine concentrations of antigen, varying from 1:5 to 1:1,280 dilutions of the stock solution containing 1 mg. of polysaccharide per milliliter. All dilution figures in the tables and charts are corrected to give the concentration of each reagent per milliliter of mixture.

TABLE I

CORRECTED GALVANOMETER DEFLECTIONS WITH SERUM DILUTED 1:100 AND VARIOUS CONCENTRATIONS OF ANTIGEN

TIME IN SECONDS	ANTIGEN DILUTION								
	1:2,560	1:1,280	1:640	1:320	1:160	1:80	1:40	1:20	1:10
10	0.2	0.3	0.0	0.3	0.5	0.9	0.9	0.8	0.7
20	0.1	0.1	0.1	0.8	2.2	5.1	4.1	2.1	1.0
30	0.1	0.1	0.3	2.2	5.0	11.1	10.0	5.0	1.7
40	0.0	0.1	0.6	3.7	7.9	16.4	16.5	8.7	2.7
50	0.0	0.1	0.8	4.9	10.7	20.4	21.5	13.0	3.8
60	0.1	0.4	1.3	6.1	12.6	23.3	25.9	17.1	5.6
75	0.1	0.5	2.2	7.6	14.9	27.0	30.9	22.7	8.7
90	0.1	0.7	2.7	8.6	16.6	29.2	34.3	26.9	11.5
105	0.1	0.8	3.4	9.4	17.8	31.0	37.0	30.2	14.3
120	0.2	1.0	3.9	9.8	19.0	32.6	39.2	32.9	16.9
150	0.5	1.5	4.7	10.7	20.7	34.9	42.1	36.8	21.1
180	0.8	1.9	5.1	11.6	22.0	36.9	45.0	39.6	24.1
210	0.9	2.1	5.7	12.0	23.1	38.5	46.7	41.2	26.1
240	0.9	2.5	5.8	12.7	23.9	39.7	47.8	42.7	27.4
270	1.0	2.8	6.1	12.9	24.8	40.8	49.0	44.1	28.2
300	1.1	2.9	6.1	13.0	25.3	41.8	50.7	44.8	29.0
Supernatant examination									
Excess antigen	−	−	−	−	−	−	+	+	+
Excess antibody	+	+	+	+	+	−	−	−	−

In Table I are listed the corrected galvanometer deflections obtained with a constant amount of serum (final dilution 1:100) and varying amounts of antigen. The data for all constant antibody series were similar. These data are plotted in Fig. 2 as deflection-time curves to show the progress of the reaction for each antigen dilution. Initially, the most rapid precipitate formation occurred in the mixture containing antigen diluted 1:80 (closed circles), the supernatant of which contained neither antigen nor antibody. With smaller amounts of antigen (region of antibody excess) the precipitate formed more slowly (crosses). The maximal five-minute reading was obtained with antigen diluted 1:40, the supernatant of which was found to contain an excess of antigen (open circles). This dilution of antigen, however, caused a slower initial rate of precipitation than that observed with the optimal antigen dilution. More concentrated antigen resulted in a further slowing of the reaction and a lower galvanometer deflection at the end of the experiment. This was confirmed by all constant antibody series in which the deflections did not exceed the limits of the galvanometer scale during the five-minute period of observation.

The crossings of the time-deflection curves in these experiments illustrate the dangers of interpreting results from a single observation at a single time interval. For example, the same dilution of serum (1:100) will give the same

reading (approximately 20) at the same time interval (approximately one hundred and forty seconds) after mixing with antigen diluted either 1:160 or 1:10. It is obvious that one must know either the shape of the curve or work only within a specified range.

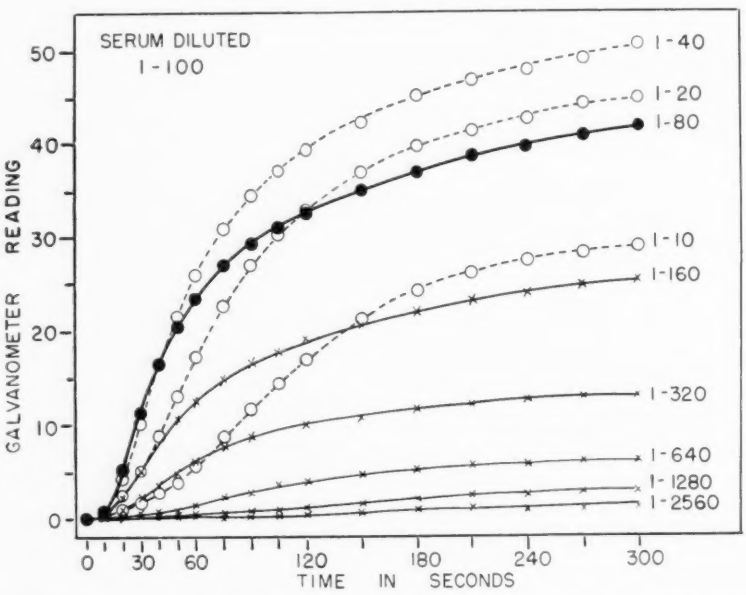

Fig. 2.—Deflection time curves obtained with constant amounts of antibody and varying dilutions of antigen over a 5-minute period. Symbols are the same as in Fig. 1.

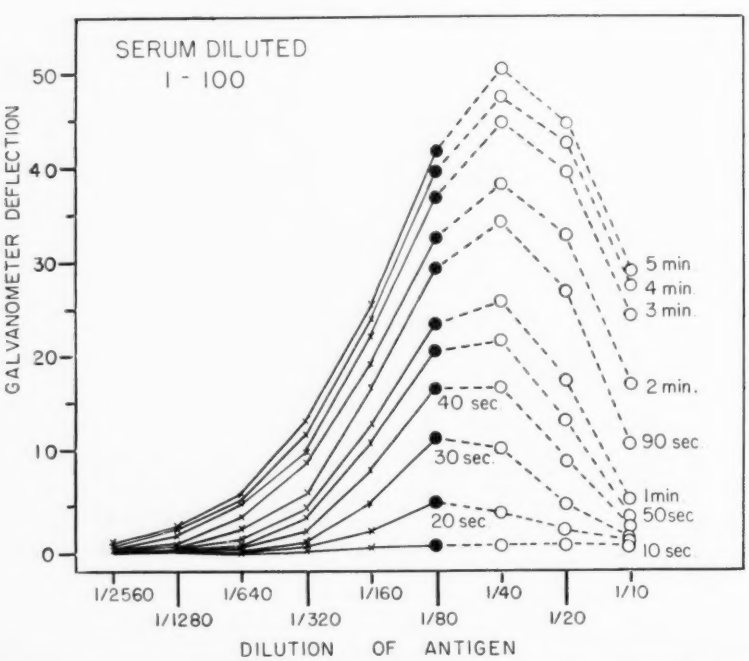

Fig. 3.—Deflection-antigen dilution curves obtained at various time intervals after mixing. Symbols are the same as in Fig. 1.

In Fig. 3 these same data are plotted more conveniently with antigen dilutions as abscissae. This shows again that the 1:80 antigen dilution precipitates fastest during the first thirty seconds. At forty seconds the 1:40 dilution has caught up with the optimal dilution, and after fifty seconds the greatest deflection is obtained with the 1:40 dilution.

TABLE II

CORRECTED GALVANOMETER DEFLECTIONS OF THE LEADING AND ADJACENT TUBES FOR VARIOUS DILUTIONS OF ANTIGEN AND SERUM AT SIGNIFICANT TIME INTERVALS

SERUM DILUTION	TIME IN SECONDS	ANTIGEN DILUTION						
		1:640	1:320	1:160	1:80	1:40	1:20	1:10
1:400	150	0.6	*1.0*	0.5				
1:282.6	40	0.7	*1.0*	0.8	0.6			
1:282.6	240		7.1	*7.2*	4.9			
1:200	40		0.5	*1.0*	0.6	0.5		
1:200	210		6.5	10.8	*11.4*	5.8		
1:141.3	40		0.3	*1.4*	1.1	0.8	0.6	
1:141.3	105		3.5	9.5	*9.9*	9.0	7.0	
1:141.3	210		8.7	17.1	20.6	*20.7*	18.6	
1:100	20			2.2	*5.1*	4.1	2.1	
1:100	40			7.9	16.4	*16.5*	8.7	
1: 70.66	10				2.1	*2.7*	2.0	0.9
1: 70.66	50				27.2	36.0	*36.4*	22.3
1: 50	10					15.0	*17.0*	9.6
1: 35.33	10					32.8	*41.3*	39.6
1: 25	10						57.1	*Off scale*

The deflections of the optimal mixtures are italicized.

The optimal ratios obtained over a fairly wide range of serum dilutions are illustrated by the data presented in Table II. A deflection of 1.0 scale unit was chosen arbitrarily as the smallest value indicating a significant difference in the amount of precipitate. The data in this table were selected to show only the deflections of the optimal and adjacent tubes at the earliest time intervals that significant differences could be measured. The deflection and shifting times of the optimal tube to the mixture containing more antigen are included. These data are plotted in Fig. 4, which shows the results of testing the supernatants for antigen and antibody and indicates the straight line relationship of the optimal tube to both the antigen dilution and the serum dilution. It illustrates also the important point that except in the region of concentrated antibody, the first tube to show definite precipitation lies in the region in which neither antigen nor antibody is present in the supernatant. The maximal five-minute deflection, however, occurs in the region of antigen excess.

Constant Antigen-Varying Antibody Series.—The data, rearranged to compare the reaction speeds obtained with constant amounts of antigen and varying concentrations of antibody, showed that no antibody dilution was optimal for any concentration of antigen. The speed of the reaction increased as the antibody concentration was increased, and in no instance did excess of antibody cause inhibition of the precipitation rate (Fig. 5).

Relationship of Antigen Concentration to Galvanometer Deflection in Region of Serum Excess.—The observations of Bukantz, Cooper, and Bullowa[15] are confirmed by the data presented in Table III, giving the five-minute readings obtained in the regions of serum excess. For antigen dilutions varying from

1:1,280 to 1:160, the galvanometer deflection was directly proportional to the antigen concentration, provided serum was greatly in excess. One unit on the scale represented an average concentration of 0.25 gamma of polysaccharide per milliliter of mixture. These data are plotted in Fig. 6, using the logarithm of the five-minute readings to show the straight line relationship between the log

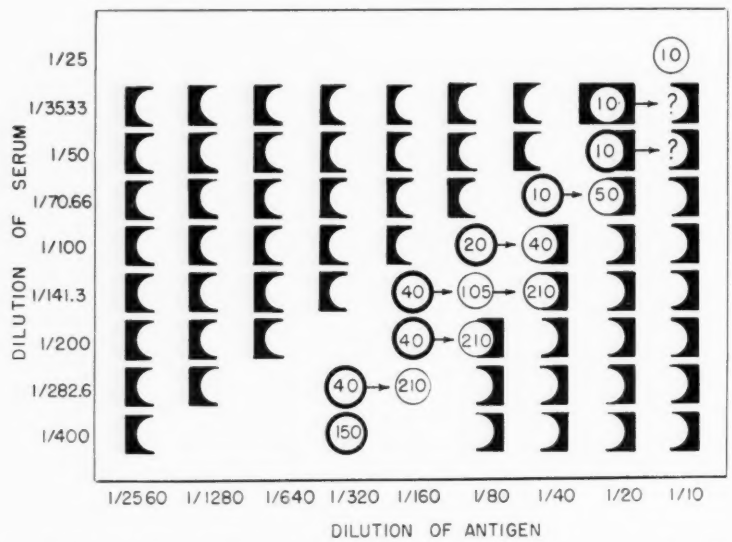

Fig. 4.—Optimal antigen dilutions for varying dilutions of serum. The figure within each circle represents the earliest time interval after mixing at which the deflection of the indicated mixture was significantly higher than deflections obtained at the same time interval with other concentrations of antigen. The results of supernatant examinations are shown by the semicircles, those on the right of the chart indicating excess antigen and those on the left, excess antibody.

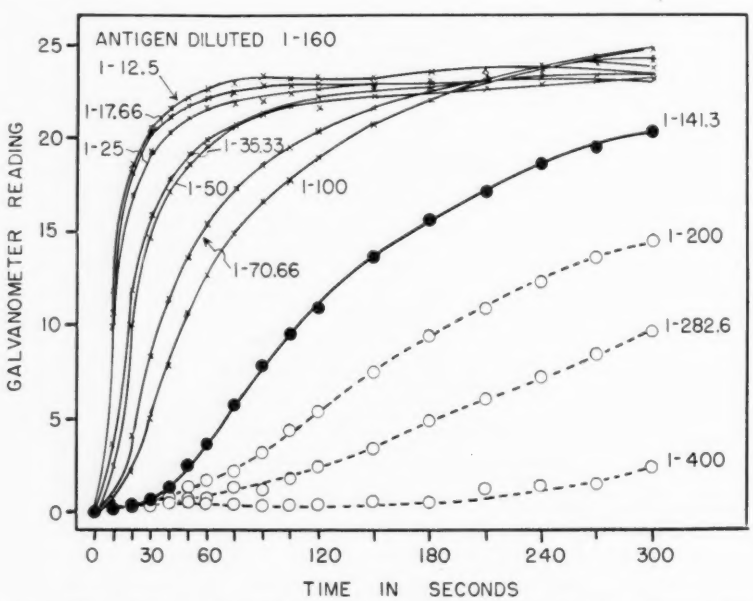

Fig. 5.—Deflection time curves obtained with constant amounts of antigen and varying amounts of antibody. Symbols are the same as in Fig. 1.

TABLE III

GALVANOMETER DEFLECTIONS FIVE MINUTES AFTER MIXING IN REGION OF EXCESS ANTIBODY

SERUM DILUTION	ANTIGEN DILUTION				
	1:1,280	1:640	1:320	1:160	1:80
1: 12.5	3.5	7.0	13.0	23.2	39.0
1: 17.66	3.1	6.2	12.6	24.0	40.1
1: 25	3.3	7.7	12.7	23.1	41.4
1: 35.33	3.2	6.5	12.6	23.5	41.2
1: 50	3.0	6.8	12.9	24.2	44.9
1: 70.66	2.7	6.2	12.5	24.8	43.6
1:100	2.9	6.1	13.0	25.3	
Mean	3.1	6.6	12.8	24.0	41.7
Weight (in gamma) of polysaccharide causing deflection of one scale division					
	0.25	0.24	0.25	0.26	0.30

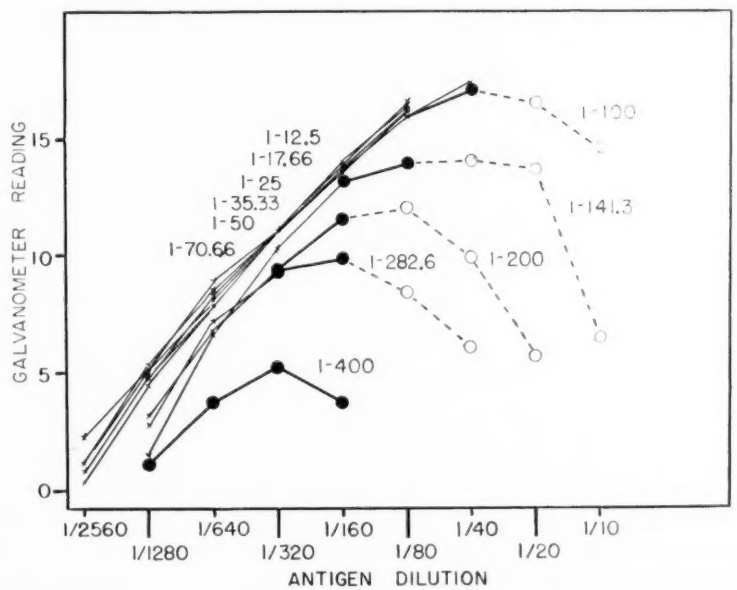

Fig. 6.—Deflection-antigen dilution curves obtained five minutes after mixing, illustrating that in the region of antibody excess there is a straight-line relationship between the galvanometer deflection at constant time and the antigen concentration. Symbols are the same as in Fig. 1.

of the deflection and the log of the antigen concentration. This chart includes also the results obtained in the region of excess antigen (open circles) and the optimal antigen concentration (closed circles). Maximal deflections within five minutes were not attained, as a rule, in the optimal mixtures, but it is possible that these mixtures might have attained the same deflection as those with excess of antibody if observations had been continued for a longer period.

DISCUSSION

No conclusions can be drawn from these experiments concerning the actual rate of reaction between antigen and antibody. The instrument records only the amount of light striking a photoelectric cell after reflection from particles precipitated during the reaction, and there is no evidence that the galvanometer deflection is a function of the weight or the volume of the precipitate. Further-

more, no significance can be attached to the shape of the deflection time curves because the increase in deflection can result from an increase in the number of particles of uniform size, increase in size of a uniform number of particles, or a summation of both factors.

Although the purpose of these experiments was to develop a procedure which could be applied to the problem of precipitin titration in complex antigen-antibody systems, the results indicate that it has certain advantages over other accepted methods of quantitative analysis. Since the optimal proportions principle is valid over a wide range of antigen-antibody dilutions, titration requires only one dilution of serum. With the refined antipneumococcus type I antibody used in these experiments, a complete titration was possible with 14.0 ml. of a 1:100 dilution of serum.

The method is not only more economic of materials but is less time-consuming, a complete run requiring approximately two hours. In comparison with the Dean-Webb procedure the photoelectric method is much more advantageous because it allows time for accurate measurement and thorough mixing of materials. It removes also any doubt concerning the optimal time for reading the reaction, since these experiments have demonstrated that the optimal ratio is determined by noting the fastest reacting mixture as early as possible in the reaction. If the reactions are compared later, the greatest deflection is found in the region of antigen excess.

The optimal ratio, as determined by this method, probably corresponds to the equivalence point described by Heidelberger, since Culbertson[16] showed that the "neutralization" point, as determined by absence of both antigen and antibody in the supernatants, agreed with the results obtained by the chemical method of analysis. A further agreement between the optimal ratio method and the chemical method is evidenced by calculation of the number of molecules of antigen and antibody reacting at the optimal point. Assuming a molecular weight of antibody to be 160,000, the number of molecules in 0.01 ml. of this serum, containing 5.7 mg. of antibody N, or 35.62 mg. antibody protein per milliliter is 13.5 by 10^{14}. The antigen dilution optimal for 0.01 ml. of serum contains 0.0125 mg. SSS per milliliter. Assuming a molecular weight of 5,000, this antigen dilution can be calculated to contain 15.1 by 10^{14} molecules of SSS per milliliter, suggesting that the antigen and antibody molecules combine in a 1:1 ratio when mixed in optimal proportions.

Culbertson[16] emphasized the great inaccuracies of the popular antigen-dilution method of precipitin titration, based upon observing the greatest dilution of antigen causing a visible precipitate with constant amounts of antibody at equilibrium. His contention that such a method was applicable to the titration of antigen content and not to the determination of antibody content is confirmed by these experiments which demonstrate that if excess antibody is present, the galvanometer deflection after a suitable time interval is dependent on antigen concentration alone. Bukantz, Cooper, and Bullowa[15] suggested that the antibody content could be estimated by observing the galvanometer deflection at a constant time interval and noting the concentration of antigen yielding the maximal deflection. However, the maximum deflection in a constant antibody

series is attained always in the region of antigen excess and, therefore, cannot be used as a measure of antibody concentration.

SUMMARY AND CONCLUSIONS

A photoelectric method for determining the relative precipitation rates obtained with various concentrations of antigen and antibody is described. The advantages of this method over the original procedure described by Dean and Webb may be summarized as follows:

1. The various dilutions of antigen and antibody can be measured accurately and mixed thoroughly.

2. The relative precipitation speeds can be determined early in the reaction at the stage which has been shown to be the optimal time for comparing the various precipitation rates.

3. The data are obtained objectively and can be recorded permanently.

From a study of eleven constant antibody-varying antigen series, using pneumococcus type I polysaccharide and antipneumococcus type I rabbit serum, it has been shown that:

1. The fastest initial precipitation occurs in the mixture in which antigen and antibody are in optimal proportions, as shown by absence of both reagents in the supernatant.

2. The maximum galvanometer deflection is obtained in the region of antigen excess.

3. The galvanometer deflection five minutes after mixing is directly proportional to the antigen concentration, provided excess antibody is present.

Comparison of the rates of reaction obtained in nine constant antigen-varying antibody series shows that no dilution of antibody is optimal for a constant amount of antigen. Progressive increases in antibody concentration result in progressively faster precipitation, excess antibody causing no inhibition of the reaction.

The close agreement of Heidelberger's "equivalence point," Culbertson's "neutralization point," and the optimal ratio, as determined by this method, are discussed.

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THE KAHN VERIFICATION TEST IN MALARIA

ALBERT DE GROAT,* CAMP RUCKER, ALA.

KAHN has recently published a verification test[1] for the detection of false positive reactions in the serology of syphilis. More recently, Kahn, McDermott, and Marcus[2] and Jacobsthal[3] have reported that this test is capable of differentiating positive serologic reactions in syphilis from those occurring in malaria. An opportunity was afforded me to apply the verification test to a group of clinically nonsyphilitic soldiers with malaria who gave positive serologic reactions for syphilis. This article will present the results obtained.

The verification test is based on the principle that syphilitic serums giving positive serologic reactions show under proper conditions more marked precipitation at 37° than at 1° C. (syphilitic type of reaction), while nonsyphilitic serums giving positive reactions show under the same conditions more marked precipitation at 1° than at 37° C. (general bio-

TABLE I

SEROLOGIC RECORD OF MALARIAL CASES

NAME	DATE	KAHN REACTIONS		QUANTITATIVE SEROLOGIC UNITS	VERIFICATION TEST (TYPE OF REACTION)
		STANDARD TEST	PRESUMPTIVE TEST		
J. Z.	10/24/41	Pos. (++++)	Pos. (++++)	200	General biologic
N. A.	11/ 3/41	Neg.	Neg.	0	General biologic
M. T.	11/ 3/41	Neg.	Neg.	0	Negative
M. J.	11/ 3/41	Neg.	Neg.	0	General biologic
T. F.	11/ 3/41	Neg.	Pos. (+++)	0	General biologic
E. H.	11/ 3/41	Neg.	Neg.	0	General biologic
W. C.	11/ 3/41	Pos. (+++)	Pos. (++++)	3	General biologic
M. K.	11/ 3/41	Neg.	Neg.	0	Negative
H. C.	11/ 3/41	Pos. (++++)	Pos. (++++)	80	General biologic
A. L.	11/ 3/41	Neg.	Neg. (+)	0	General biologic
R. W.*	11/ 3/41	Neg.	Neg.	0	General biologic
W. J.	11/ 3/41	Neg.	Neg.	0	Negative
C. A.	11/ 3/41	Neg.	Pos. (+++)	0	General biologic
J. Q.	11/ 3/41	Pos. (++)	Pos. (++++)	2	General biologic
R. M.	11/ 3/41	Doubt. (+)	Pos. (++++)	1	General biologic
D. L.*	11/ 7/41	Pos. (++++)	Pos. (++++)	40	General biologic
E. G.	11/ 7/41	Pos. (++++)	Pos. (++++)	40	Inconclusive
A. D.	11/14/41	Pos. (++++)	Pos. (++++)	4	General biologic

*Nonmalarial cases; see Table IV.

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logic, nonsyphilitic, type of reaction). Serums giving negative serologic reactions generally show no precipitation at 37° or 1° C., and the verification reaction is referred to as negative type. When precipitation of equal strength is obtained at these two temperatures, the verification results are referred to as inconclusive.

Blood specimens from the seropositive cases of malaria and also from some seronegative cases were sent to Doctor Kahn's laboratory at the University of Michigan Hospital for verification tests. No indications as to the source of these specimens were submitted. The serologic reports received from that laboratory are summarized in Table I. Of eighteen serums examined, the standard Kahn test proved to be positive in seven, doubtful in one, and negative in the remaining ten. The supersensitive presumptive test gave ten positive reactions in the same group of serums. All these positive reactions, according to the verification test, were of the general biologic (nonsyphilitic) type, except in one instance in which the result of the verification test was reported as inconclusive.

TABLE II

KAHN VERIFICATION TEST IN SEROPOSITIVE CASES OF MALARIA

DATE	KAHN REACTIONS		QUANTITATIVE SEROLOGIC UNITS	VERIFICATION TEST (TYPE OF REACTION)
	STANDARD TEST	PRESUMPTIVE TEST		
		Case 1 (J. Z.)		
10/24/41	Pos. (++++)	Pos. (++++)	200	General biologic
11/ 3/41	Pos. (++++)	Pos. (++++)	80	General biologic
11/ 7/41	Pos. (+++)	Pos. (++++)	3	General biologic
11/15/41	Neg.	Neg.	0	General biologic
		Case 2 (H. C.)		
11/ 3/41	Pos. (++++)	Pos. (++++)	80	General biologic
11/ 7/41	Pos. (+++)	Pos. (++++)	3	General biologic
12/ 6/41	Neg.	Neg.	0	General biologic
12/31/41	Neg.	Neg.	0	Negative
		Case 3 (W. C.)		
11/ 3/41	Pos. (+++)	Pos. (++++)	3	General biologic
11/ 7/41	Neg.	Pos. (+++)	0	General biologic
11/14/41	Neg.	Neg.	0	General biologic
12/31/41	Neg.	Neg.	0	Negative
		Case 4 (J. Q.)		
11/ 3/41	Pos. (++)	Pos. (++++)	2	General biologic
11/ 7/41	Pos. (++)	Pos. (++++)	2	General biologic
11/27/41	Neg.	Neg.	0	Negative
12/ 4/41	Neg.	Neg.	0	Negative
		Case 5 (R. M.)		
11/ 3/41	Doubt. (+)	Pos. (++++)	1	General biologic
11/ 7/41	Neg.	Doubt. (++)	0	General biologic
12/ 6/41	Neg.	Neg.	0	Negative
		Case 6 (E. G.)		
11/ 7/41	Pos. (++++)	Pos. (++++)	40	Inconclusive
11/14/41	Neg.	Pos. (++++)	0	General biologic
12/ 4/41	Neg.	Neg.	0	Negative
12/11/41	Neg.	Neg.	0	Negative
		Case 7 (A. D.)		
11/14/41	Pos. (++++)	Pos. (++++)	4	General biologic
11/25/41	Pos. (+++)	Pos. (++++)	3	General biologic
1/30/42	Neg.	Neg.	0	Negative

Of interest is the fact that five of the negative reactions with the standard and presumptive Kahn tests were also, according to the verification test, of the general biologic (nonsyphilitic) type. Generally, serums that give negative reactions with serodiagnostic tests give the negative type of reaction with the verification test. The general biologic type of reaction given by these negative reacting serums indicates, according to Kahn, that these serums contain nonsyphilitic "antibody" and that they might give positive reactions with certain serodiagnostic tests and, under certain conditions, with perhaps all tests.

Of interest is the fact also that, according to Table I, malarial serums may give high titers with the quantitative Kahn reaction. Two of the serums gave a titer of 40, one of 80, and one of 200. Evidently positive reactions in malaria are not limited to low serologic titers.

The next problem that presented itself was to find if the general biologic (nonsyphilitic) type of reactions obtained in the patients with malaria could be accepted as serologic evidence of the absence of syphilis in these cases. Repeated serologic examinations of the blood specimens in these cases, it was believed, would throw light on this problem. Thus, positive serologic reactions in acute malaria generally become negative after recovery. Would the positive reactions in the patients under investigation persist or would they become

TABLE III

KAHN VERIFICATION TEST IN CASES OF MALARIA, ESSENTIALLY SERONEGATIVE

DATE	KAHN REACTIONS		QUANTITATIVE SEROLOGIC UNITS	VERIFICATION TEST (TYPE OF REACTION)
	STANDARD TEST	PRESUMPTIVE TEST		
		Case 8 (T. F.)		
11/ 3/41	Neg.	Pos. (+++)	0	General biologic
11/ 7/41	Neg.	Neg.	0	General biologic
11/14/41	Neg.	Neg.	0	General biologic
11/21/41	Neg.	Pos. (++++)	0	General biologic
11/29/41	Neg.	Neg.	0	General biologic
1/16/42	Neg.	Neg.	0	Negative
		Case 9 (C. A.)		
11/ 3/41	Neg.	Pos. (+++)	0	General biologic
11/14/41	Neg.	Neg. (+)	0	General biologic
11/21/41	Neg.	Neg.	0	General biologic
11/28/41	Neg.	Neg.	0	General biologic
12/ 4/41	Neg.	Neg.	0	General biologic
12/11/41	Neg.	Neg.	0	Negative
		Case 10 (A. L.)		
11/ 3/41	Neg.	Neg. (+)	0	General biologic
11/ 7/41	Neg.	Neg. (+)	0	General biologic
11/25/41	Neg.	Neg.	0	General biologic
1/ 7/42	Neg.	Neg.	0	General biologic
		Case 11 (E. H.)		
11/ 3/41	Neg.	Neg.	0	General biologic
11/ 7/41	Pos. (++)	Pos. (++++)	2	General biologic
11/15/41	Neg.	Neg.	0	General biologic
11/25/41	Neg.	Neg.	0	Negative
12/ 2/41	Neg.	Neg.	0	Negative
		Case 12 (M. J.)		
11/ 3/41	Neg.	Neg.	0	General biologic
11/21/41	Neg.	Neg.	0	General biologic
12/ 4/41	Neg.	Neg.	0	Negative

negative with the subsidence of the fever and with recovery? Accordingly, arrangements were made for the serologic follow-up of these patients.

Table II lists seven seropositive cases of malaria which became seronegative in about one to three months. The verification reactions in these patients changed from the general biologic type to the negative type of reactions. An exception is Case 1 in which the original general biologic type of reaction persisted. Table III lists five cases of malaria, essentially seronegative, except that with the supersensitive presumptive test, seven positive reactions of varying degree were obtained through the period of investigation. In these cases also, the verification test at first gave the general biologic type of reactions, and later, the negative type.

Two febrile patients of the original group of twenty-one, listed in Table IV, were found not to have malaria. One patient with cellulitis (Case 13) was Kahn-negative. Another (Case 14), with an acute respiratory infection, was Kahn-positive. The verification test, in both cases, gave the general biologic type of reactions. On repeated serologic examinations, the Kahn-positive reaction rapidly became negative. But in both cases, the general biologic type of verification reactions persisted, although the patients had recovered. It was impossible to continue verification studies in these patients due to the transfer of the soldiers from Fort Custer.

TABLE IV

KAHN VERIFICATION TEST IN TWO NONMALARIAL CASES

DATE	KAHN REACTIONS		QUANTITATIVE SEROLOGIC UNITS	VERIFICATION TEST (TYPE OF REACTION)
	STANDARD TEST	PRESUMPTIVE TEST		
Case 13 (R. W.); Cellulitis, Chills, and Fever				
11/ 3/41	Neg.	Neg.	0	General biologic
12/ 4/41	Neg.	Neg.	0	General biologic
1/ 7/42	Neg.	Neg.	0	General biologic
Case 14 (D. L.); Acute Respiratory Infection				
11/ 7/41	Pos. (++++)	Pos. (++++)	40	General biologic
11/14/41	Neg.	Neg. (+)	0	General biologic
11/21/41	Neg.	Neg.	0	General biologic

SUMMARY

The Kahn verification test was applied to a group of clinically nonsyphilitic soldiers with malaria giving positive serologic reactions for syphilis. It was found that these soldiers gave the general biologic (nonsyphilitic) type of reactions, indicating that the verification test can be employed as an aid in establishing the differential diagnosis of malaria and syphilis.

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DETERMINATION OF BLOOD PRESSURE IN RATS BY DIRECT OBSERVATION OF BLOOD VESSELS*

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ALTHOUGH several methods of determining blood pressure in small animals have been described, certain complicating features in the technique of each of them often render them inapplicable to the experimental problem on hand. It is the purpose of this communication to point out briefly the essential features of previously described methods and to present a simple method for repeated determinations which produces no trauma or loss of blood and which may be used on either anesthetized or unanesthetized animals.

Methods involving direct cannulation of large vessels require either local or general anesthesia. Trauma and loss of blood result to a greater or lesser degree during exposure and cannulation of the vessel, and in most of the methods of this type the animal is usually sacrificed. The method of Woodbury and Hamilton[1] has definite advantages in some problems but is not simple technically, the apparatus is expensive, and each determination requires surgical exposure of the vessels to be punctured. The values obtained by these investigators are higher than pressures obtained, also by arterial cannulation, by Rous and Drury[2] and by Chanutin and Ferris.[3] Diaz and Levy[4] described a method for determining blood pressure in rats which depends upon the flow of blood from the incised tail when the pressure in the arteries exceeds that in a pneumatic cuff about the base of the animal's tail. The values obtained by these authors coincided rather closely with pressures obtained by aortic cannulation. This method produces loss of blood and trauma with each determination, and where frequent determinations are made, this degree of blood loss may complicate certain types of experiments.

Indirect methods which necessitate no trauma or loss of blood have been described by several authors. Kunstmann[5] and Bonsmann[6] made use of the restoration of pink color due to the return of blood to a limb or tail which has been previously emptied of blood by a tight bandage. The flow of blood is controlled by means of a compression cuff at the base of the animal's tail. The values obtained by both these investigators are lower than the values reported by other investigators. Williams, Harrison, and Grollman[7] have described a practical and accurate method which is based on plethysmographic measurement of the increase in volume of the rat's tail when the pressure in the arteries exceeds that in a compression cuff about the animal's tail. This method is used on trained rats and requires preliminary warming of the animal for accuracy. A similar method was described by Byrom and Wilson.[8]

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Two methods have been described which involve the application of a compression cuff to the thigh and observation of the flow of blood in the small blood vessels of the foot. Griffith[9] observed microscopically the cessation and restoration of flow in the small superficial vessels on the dorsum of the rat's foot. The observations were made with strong reflected light on an area that had been depilated and cleared with immersion oil. He recommended that several fields be observed because of the uncertainty of the type of vessels under observation. We were unable to obtain consistent results by this method. McMaster[10] reported a similar method in which flow in the blood vessels at the base of the claws of mice was observed by transmitted light. He pointed out that similar observations cannot be made in rats, even in young ones.

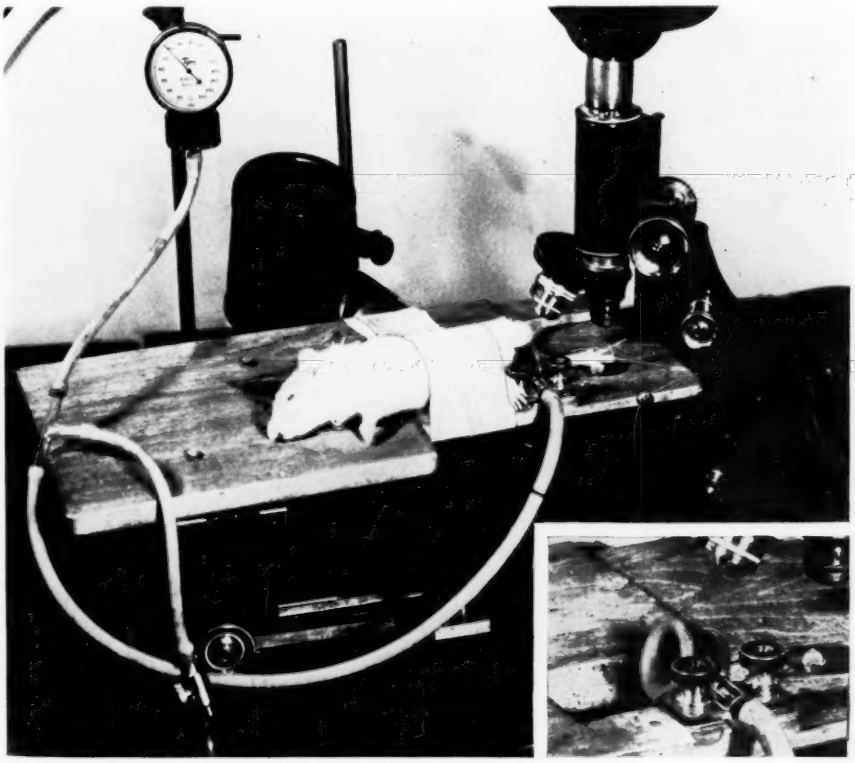

Fig. 1.—Arrangement of apparatus for determining blood pressure of rat. Insert shows details of pressure cuff and transillumination cone.

The method to be described is also based upon direct observation of blood vessels. The vessels are those of the translucent interdigital web and are observed by means of transmitted light. These vessels of the spread-out web are clearly visible so that the different types of vessels can be easily identified.

Normal rats weighing from 130 to 200 Gm. were used. The apparatus, shown in Fig. 1, consists of a cuff, similar to that described by Diaz and Levy[4] and Byrom and Wilson,[8] which is placed about the thigh. The cuff is made of a 2 cm. length of glass tubing 2 cm. in diameter and flanged on both ends, with a short length of 4 mm. glass tubing fused to the side for connection with a manometer and a rubber pressure bulb. A piece of 5/8 inch Penrose tubing

is placed inside the larger glass tube, everted over the flanged ends, and cemented to the outer surface. The cuff is attached to a board 5 by 12 inches in such a position that it rests about four inches from the end. The unanesthetized rat is kept fairly quiet by placing it in a snugly fitting glass cylinder on the end of the board carrying the cuff. One hind limb is drawn through the cuff to the level of the upper thigh and held in place by small clamps attached to the claws or by strings tied about the distal ends of two adjacent digits. The position of the foot is such that the spread-out interdigital web rests upon the upper surface of truncated glass cone fixed through the board. The cone measures approximately 1 cm. at its base, and its upper end is ground to a triangular cross section which measures approximately 0.25 cm. on each side and fits closely between the outspread digits. The web is rendered translucent by removal of the epidermal scurf by a brief application of depilatory cream and clearing with almond oil or paraffin oil. A small piece of cover slip placed upon the web serves to hold the film of oil in place and thus an even surface for observation is presented. The board with the animal in place is mounted on the stage of the microscope so that the truncated cone of glass lies in the optical axis. Light is obtained from a Leitz low voltage microscope lamp or any equivalent source. With a magnification of 100 times the vessels and the flow of blood can be clearly seen. The small arterial vessels can be differentiated from corresponding veins by their relatively thicker walls and more rapid rate of flow and by the direction of flow.

In making a determination the cuff is inflated to a pressure sufficient to stop all flow of blood. The pressure is then slowly lowered until the first continuous flow becomes visible in the arteriole under observation. The pressure at that time is taken as the blood pressure in the large arteries of the thigh. Other vessels in the field are checked to ensure accuracy.

The normal blood pressures of different animals of the unanesthetized group ranged from 90 to 140 mm. Hg. The range of pressures in different animals of the anesthetized group was from 70 to 120 mm. Hg. Repeated determinations were made on several animals of each group and were found to agree within 4 mm. Hg in any given animal. The accuracy of the method was checked by obtaining pressures by aortic cannulation on six rats immediately following determinations by the interdigital web method. The values checked within 5 mm. Hg.

SUMMARY

The arterial blood pressure of rats may be rapidly and easily determined by microscopic observation of the flow of blood in the small arterial vessels of the interdigital web. Values obtained by this method agree with pressures determined by aortic cannulation and with those reported in the literature.

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A METHOD FOR OBTAINING RECORDS OF THE TORTOISE SINUS WITH DATA ON EXTRASYSTOLES AND LENGTH OF CYCLES FOLLOWING STIMULATION

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DURING the last several years graphic records taken from the beating sinus venosus of the tortoise have been used in discussing with medical students the refractory period, extra systole, and compensatory pause of the heart. These phenomena in the ventricles and to some extent in the auricles are common descriptions in most textbooks, and a discussion of excitations near the great veins or sinoauricular node is nicely presented by Wiggers.[1] Graphic records taken from the sinus are not shown in any of the present textbooks, nor have we found them in any of the literature so far reviewed. It seems worth consideration, therefore, to describe briefly the method used to obtain records of the sinus venosus and to exhibit some of the records obtained. The literature is, for the most part, of long standing, and excellent reviews are available.[2, 3] These and original contributions having a direct bearing on the problem are discussed briefly as their citation appears in the body of this paper.

PROCEDURE

Twenty-seven pithed tortoises about six inches in shell length were used, and from these about two thousand records of sinus stimulation were saved for study. The usual method of recording the auricular and ventricular contraction by means of aluminum heart levers was employed. In order to obtain tracings of the beating sinus, fine thread was usually attached directly to the sinus (5 to 8 mm. to the right of the midline) with the aid of fine-pointed dissecting forceps. In a few cases the thread was placed around the large veins to the right of the sinus. The heart lever required careful counter balance and adjustment, but when so arranged usually gave a clear tracing of the sinus beats. By means of a small, bent glass rod with a hook at the end the base of the ventricle could be adjusted to the left and ventrally in such a way

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as to free the sinus tracing from auricular and ventricular interference. One stimulating electrode of fine copper wire was placed on and sometimes directly into the wall of the sinus, and the second electrode was stuck in the liver one inch back from the heart and on the midline. Make-and-break stimuli from a Harvard inductorium were given. Best results were obtained if the heart rate was slow, so in six experiments the rates were slowed by placing the shell on ice during the entire experiment. A depression in a large cake of ice was chipped out to fit the contour of the shell and, when the tortoise was placed in this, satisfactory slowing of the heart rate soon followed.

RESULTS

Tracings taken simultaneously from the sinus, auricles, and ventricles show the normal sequence of activity in these respective parts as the contracting wave moves over the heart.

Fig. 1*B* shows that stimulation of the sinus in the middle third of the cycle with a relatively weak stimulus (make) has no effect on the rhythm, while the break stimulus applied at the same time of the cycle produced an extra con-

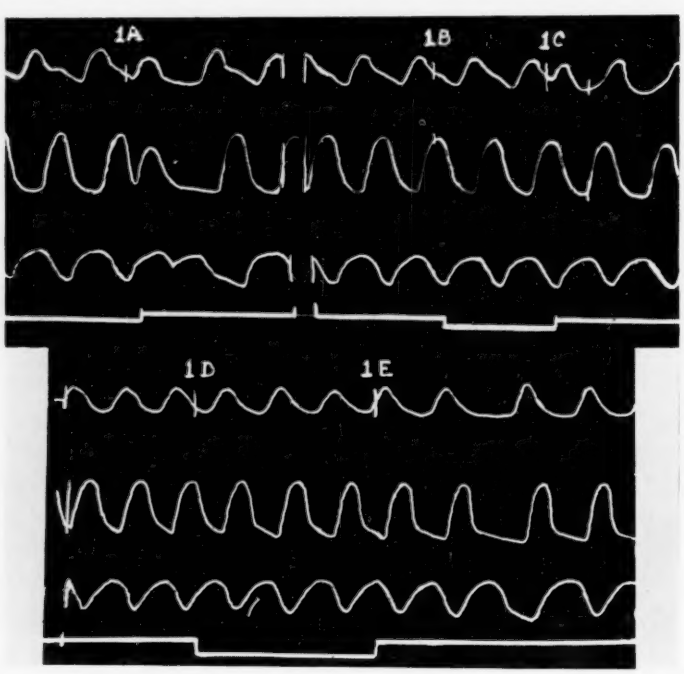

Fig. 1.—Heart rate, 17 per minute. Tracings from above downward: sinus, auricles, ventricles, signal magnet (down stroke make stimulus, up stroke break stimulus). Arrows show where the stimulus was applied to the sinus (one dry cell, Harvard coil, 4 cm.).

A, A stimulus applied to the sinus in the last third of diastole induced an extrasystole. The pause following is longer than normal. The "postcompensatory" contraction of the sinus is higher than normal.

B, A make stimulus, just below threshold for this tortoise, applied to the sinus in the middle third of the cycle had no effect.

C, The break stimulus applied in the same phase of the cycle as in *B* was followed by an extrasystole and a pause of shorter duration than normal. The "postcompensatory" contraction of the sinus is slightly higher than normal.

D, A make (in this case subthreshold) stimulus applied in the last third of the cycle had no effect.

E, Since the make stimulus as in *D* was subthreshold, the break stimulus as applied was relatively weak, yet the disturbance induced by stimulation in the refractory period caused the next three pauses of the sinus to be longer.

traction in the auricles and ventricles (Fig. 1*C*). In this case the extra contraction of the sinus was followed after a pause of shorter duration than normal by the usual rhythmic sinus discharges. Lewis[3] describes sinus extrasystoles as rare in the human subject but points out that the returning cycle may be shorter than normal. Engelmann[4] showed in amphibians that an extrasystole induced by stimulating the sinus was followed by returning cycles of the same length as the initial cycles. In our experience if the stimulus is applied to the sinus in the middle half of the cycle, the pause following the extrasystole may be of normal length about 20 per cent of the time or, using the expression of

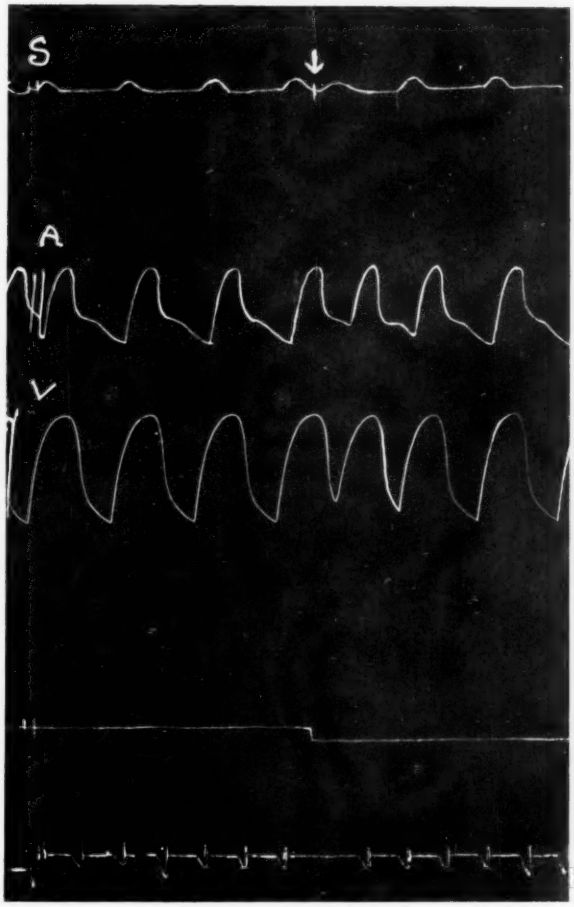

Fig. 2.—Time in 5 seconds, otherwise tracings and coil same as in Fig. 1. A stimulus applied at the beginning of the middle third of the cycle induced an extrasystole of the sinus which was carried to the auricles and ventricles. This induced cycle was approximately equal in length to the preceding cycle.

Lewis, the returning cycle is approximately equal in length to the initial cycles (Fig. 2). On the other hand, the next discharge of the sinus, after the extrasystole, may be delayed as compared to initial cycles or subsequent returning cycles (Figs. 1*A*, 3 *A* and *B*, 4*B*). Cushny,[5] working with the isolated ventricle of mammals, introduced the idea of fatigue of the pacemaker due to extra impulses either from artificial stimuli or "impulses reaching it from the

Keith-Flack node." Lewis[3] has discussed this point in regard to the sinoauricular node and states that "The manner and circumstances in which this depressant effect of an extrasystole is exerted are not yet entirely clear."

In order to study factors which might determine the length of the pause following an extrasystole of the sinus, 772 records were carefully measured with the aid of compass and lens. This study revealed that a stimulus applied to the sinus during contraction (refractory period) always failed to cause an

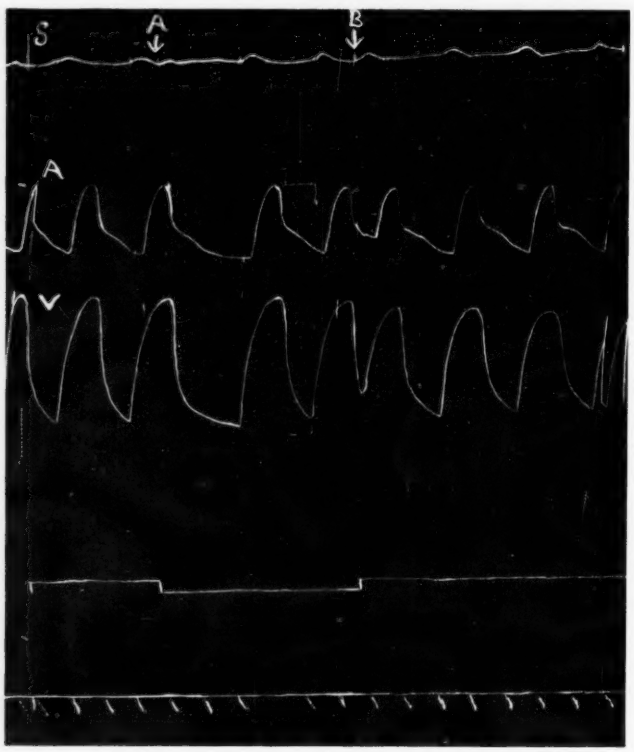

Fig. 3.—Time in 5 seconds, otherwise tracings and coil same as in Fig. 1.

A, Stimulus applied to the sinus at the beginning of the middle third of the cycle. Extra contraction of the sinus not carried to the auricles and ventricles. "Postcompensatory" contraction of auricles and ventricles higher than normal. Induced cycle slightly longer than the preceding cycle.

B, Stimulus applied in the middle third of the cycle. Extrasystole of the sinus carried to auricles and ventricles, followed by a pause of longer duration than normal.

extra contraction but the rhythm may be disturbed. In about one-fourth of the records the next pause was longer (Table I), and sometimes this disturbance was evident in the next two or more following cycles (Fig. 1*E*). A stimulus of at least threshold strength for the sinus but applied during the relaxation of the sinus (descending limb of the record) often induced a change in rhythm, and the subsequent pause was altered, being shorter in a few records (3.4 per cent), normal in 53.0 per cent, and longer in 43.6 per cent (Table I). This period may be looked upon as the relative refractory period and variable in length as subsequent discussion will show.

A stimulus applied in the first quarter of the cycle usually gave no extrasystole, but in a few cases where an extrasystole occurred the pause that

followed was always of shorter duration than normal. Table I shows a summary of nineteen records of tortoises in which this type of response occurred. A stimulus falling within the first third of the cycle gave an extrasystole which was followed in 77.1 per cent by a pause of shorter duration than normal and in 9.4 per cent by a longer pause than normal (Table I). A stimulus applied during the middle third of the cycle gave an extrasystole and a pause which was shorter than normal in 54.1 per cent. In contrast to these examples, a stimulus given in the last third of the cycle caused as expected an extrasystole, but the pause that followed was usually (80.0 per cent) longer than normal (Table I).

TABLE I

The length of 772 cycles following stimulation is compared to the previous cycle. In records of tortoises in which the stimulus was not applied in the refractory period or relative refractory period, the following cycles included an extrasystole in each case.

STIMULATED IN:	NO. OF RECORDS	LENGTH OF NEXT CYCLE FOLLOWING EXTRASYSTOLES OR STIMULATION		
		SHORTER	SAME	LONGER
Refractory period	100	0	77	23
Relative refractory period	87	3 (3.4%)	46 (53.0%)	38 (43.6%)
First quarter of cycle	19	19	0	0
First third of cycle	96	74 (77.1%)	13 (13.5%)	9 (9.4%)
Middle third of cycle	270	146 (54.1%)	56 (20.7%)	68 (25.2%)
Last third of cycle	200	17 (8.5%)	23 (11.5%)	160 (80.0%)

Thus it is clear that the length of the pause that follows an extrasystole in the sinus of the tortoise is not fixed but closely related to the time in the cycle when the stimulus was applied (Figs. 1 to 4). The earlier in the cycle that an effective stimulus is applied, the shorter the pause is likely to be, and conversely a stimulus applied in late diastole of the sinus is almost certain to be followed by a pause of longer duration than normal. At least two factors have been found that tend to change these expected results. The first of these deals with the relative refractory period of the sinus which, in our experience, varied in length in different tortoises. When this period is long, it is most difficult to obtain an extrasystole sufficiently early in the cycle to give the expected pause of shorter duration. Fig. 4*A* illustrates this very nicely and shows that a make stimulus applied in the middle third of the cycle had no effect. After a pause of a few seconds a second record was taken, and the same strength of stimulus applied slightly later in the cycle induced an extrasystole which was followed by a pause of longer duration than normal (Fig. 4*B*). In other words, it was impossible to get a reaction in the first third of the cycle with a stimulus which was considerably above threshold. The second factor also tends to fix the length of the pause but operates when the stimulus is applied in late diastole of the sinus. In these records the induced or extrasystole occurred at the expected time of the normal contraction and was followed by a pause of normal length.

The disturbance in the sinus induced by stimulation may be followed by a delay of the next rhythmic discharge, even though the artificial stimulus may have been so weak that the sinus contracted only slightly (Fig. 3*A*). This record also shows that the stimulus was either too weak to be effectively transmitted to the auricles or the auricles had not recovered sufficiently to respond,

the result being a pause in auricular and ventricular activity until the next sinus discharge, which was itself delayed. The strength of the stimulus has some effect on the length of the pause that follows an extrasystole. If the stimulus is very strong, the pause is likely to be modified in the direction of longer length. However, careful check of many records shows that the strength of the stimulus within reasonable limits does not alter the expected results in regard to the length of the pause that follows the extra contraction.

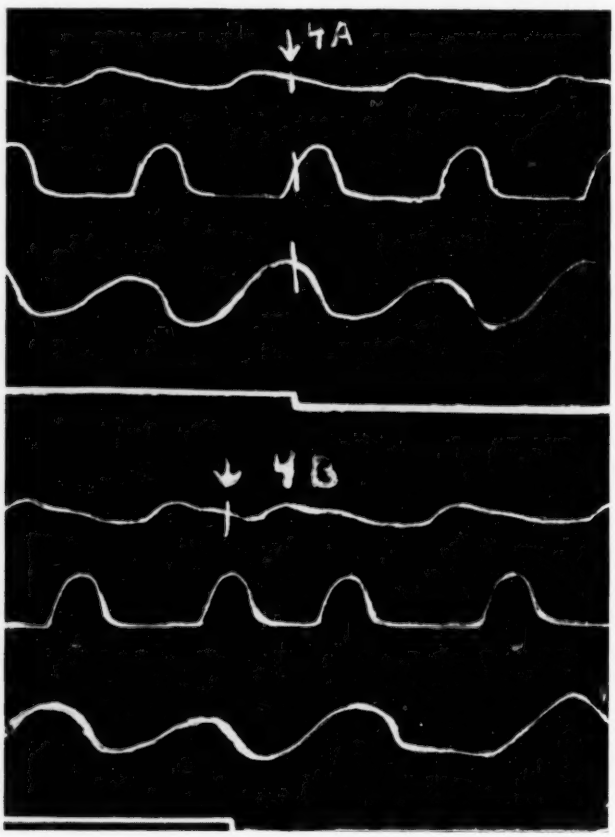

Fig. 4.—Heart rate, 21 per minute. Tracings and coil same as in Fig. 1.

A, A stimulus applied in the middle of the cycle had no effect. The relative refractory period of the sinus in this tortoise was longer than usual.

B, A stimulus of the same strength as in *A* applied slightly later in the cycle induced an extra contraction of the sinus which was carried to the auricles and ventricles. The pause that followed was longer than normal.

The increased height of the "postcompensatory" contraction as applied to the ventricle was well discussed by Cushny,[5] who compares it to a staircase phenomenon. In our work it has occurred after sinus stimulation. Fig. 3*A* exhibits it in the ventricle and to some extent in the auricle, while Fig. 1 *A* and *C* shows it occurring in the sinus even though the pause following the extrasystole was either longer or shorter than normal. Fig. 3*A* is of further interest because the compensatory pause in the auricles and in the ventricles was not preceded by an extrasystole, and therefore the factors leading to the increased height of the "postcompensatory" contraction must have their genesis associated only with the rest period.

DISCUSSION

Among the great number of papers reviewed, those dealing with electrical stimulation of the heart[6-8] have presented a mass of material and illustrations that bear on our results, yet they do not show records of sinal contraction. The original figures of Marey[9] showing the compensatory pause of the ventricle are still the classical illustrations in this field. Schultz[10] has studied records of the isolated sinus and auricles. With these researches as a background, Lewis[3] discussed and showed diagrammatically the condition of the sinus, auricles, and ventricles during the extra discharges and other irregularities of the pacemaker. Our records taken simultaneously from the sinus, auricles, and ventricles confirm and extend the diagrams of Lewis[3] and others.[11-13]

All the records shown (Figs. 1 to 4) were obtained several times so that we feel that the results illustrated by these figures are not incidental to the technique but represent the actual activity of the sinus. Also we have obtained at room temperature reproductions of all the records obtained with cooled preparations, thus demonstrating that the results obtained with cooled hearts were not bizarre due to the cooling of the heart as Cushny[5] long ago warned against.

One of the most frequent questions asked by students is what happens to the rhythmic discharge of the sinus following an extra stimulus and systole of the sinus? We have answered this by careful measurements of time relations and can therefore say that the time in the cycle when the stimulus is applied usually determines the length of the pause preceding the next sinus discharge. The next most important factor is the condition of the tortoise. All of the records illustrated as well as the data in Table I were taken from fresh preparations in which relatively weak stimuli gave satisfactory results. In addition, many records were taken from tortoises in poor condition; that is, several hours after pithing, preparing, and use. These data showed the refractory and relative refractory periods were longer than normal and that an extrasystole placed as early in the cycle as the sinus would permit was almost always followed by a longer pause than normal.

Thus it is obvious that the pause following an extra stimulus to the sinus, although variable, is predictable in length and probably depends somewhat upon the physiologic condition of the cells responsible for the discharge but mostly on the time of stimulation. With these factors in mind the stimulus may be placed so as to give an extrasystole and pause which will show a fairly consistent pattern when repeated records are taken from the same tortoise.

SUMMARY

Graphic records of the contractions of the sinus venosus of turtles were obtained simultaneously with contractions of the auricles and ventricles.

An electrical stimulus applied during the refractory period of the sinus will not cause an extrasystole, but the rhythm may be disturbed.

Electrical stimuli applied to the sinus during its quiescent period caused a contraction which may or may not be carried in turn to the auricles and ventricles.

The pause in the sinus (returning cycle) following an extra or induced contraction of the sinus may be shorter, longer, or equal to normal. The factors determining the length of this pause are governed to some extent by the physiologic condition or property of the cells in the sinus but mostly by the time in the cycle when the extrasystole is induced. With these factors in mind, the sinus of any tortoise will exhibit somewhat predetermined temporal discharges following electrical stimulation. This was indicated by the fact that an extrasystole occurring in early diastole was usually followed by a pause of shorter duration than normal, while one occurring near the end of the cycle was usually followed by a pause of longer duration than normal.

The height of the next contraction of the sinus after an extrasystole of the sinus may be higher than normal even though the pause preceding it was shorter than normal.

The strength of the stimulus may be changed from threshold to considerably above threshold without affecting the results.

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CHEMICAL

A CONVENIENT ARRANGEMENT FOR RAPID DIALYSIS*

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THE apparatus in Fig. 1 is designed (1) to give a large amount of membrane surface per cubic centimeter of solution to be dialyzed, and (2) to clear away efficiently the material which diffuses through the membrane. It is inexpensive and there are no difficult technical problems to consider.

The material to be dialyzed (*A*) is placed in a seamless cellulose dialysis tube (*B*) (Visking Corporation, Chicago). Most of the space in this tube is filled with a cylindrical glass plug (*C*), which is made from ordinary glass tubing. If the dimensions of the latter are properly selected, the liquid to be dialyzed will be evenly distributed in thin layers over a large area of membrane

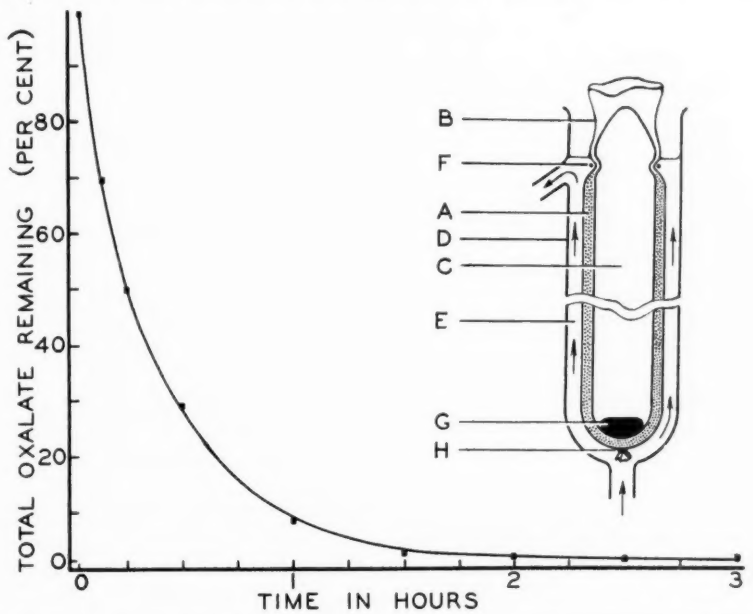

Fig. 1.—Rate of dialysis when a 2 per cent oxalic acid solution is dialyzed against water. *A*, Solution to be dialyzed; *B*, dialysis membrane; *C*, glass plug; *D*, outer glass cylinder; *E*, solution to be dialyzed against; *F*, rubber band; *G*, mercury weight; and *H*, half hitch knot at end of dialysis membrane. Drawing made by H. Kritzler.

surface. The molecules will have only a short distance to travel and the proportionately large surface area supplies an abundant number of pores. The plug is suitably weighted, with mercury for example, and the entire arrangement can be floated in water or any other solution against which one wants to dialyze. To facilitate clearing away material which diffuses through the membrane, the

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float is confined to a cylinder (*D*) through which a constant flow of liquid (*E*) can be maintained by gravity.

Dimensions of the assembly are arbitrary and within limits can be varied considerably, especially in length. Considerable use has been made of one suitable for dialyzing 25 c.c. of material. The outside dimensions of the cylindrical plug (*C*) are 1.91 × 21.5 cm., and the diameter of the dialysis membrane is listed as 29/32 inch inflated diameter by the manufacturer. In operation the thickness of the material to be dialyzed is about 0.16 cm., and each cubic centimeter is exposed to a surface area of approximately 6.0 sq. cm.

To illustrate (curve on Fig. 1) the characteristics of the apparatus with the above dimensions 25 c.c. of a 2 per cent solution of oxalic acid were dialyzed against water. The water flow through the cylinder D was 100 c.c. per minute. At the end of fifteen minutes half of the oxalate had dialyzed through the membrane, most of it had disappeared within the hour, and only small amounts remained at the end of three hours. Without the glass plug approximately 10 per cent will remain at the end of two hours. If the water flow through the cylinder is reduced to 50 c.c. per minute or increased to 600 c.c. there is no appreciable change in the dialysis rate.

ROUTINE ANALYSIS OF URINARY CALCULI*

A Rapid, Simple Method Using Spot Tests

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URINARY stone analysis is an important factor in determining the etiology of a stone and subsequent management of the patient. The common "systems" of stone analysis have often been regarded with misgivings since they appear to be too complicated, time-consuming, or otherwise unsatisfactory from the standpoint of the urologist. We have, therefore, compiled simple chemical tests whereby a dependable analysis may be made in the ordinary office laboratory in a few minutes by a worker without special training. A further advantage is that several calculi may be examined simultaneously without additional effort.

Reagents.—The appended list of reagents includes several which are not essential, since alternative methods are given to allow for individual choice. If these solutions are kept in dropper-type bottles, their use is facilitated, since only minute amounts are needed for testing.

Uric acid reagent: Consult textbooks for the preparation of the Benedict or Folin-Denis reagents used for urine and blood uric acid determinations.

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Sodium carbonate: 20 per cent solution.

Ammonium molybdate: Several different solutions may be employed but that suggested by Seifter and Trattner[10] is excellent. Dissolve 3.5 Gm. of ammonium molybdate in 75 c.c. of water and pour into 25 c.c. of concentrated nitric acid.

Manganese dioxide: A heavy, fine, black powder, MnO_2.

Resorcinol or resorcin: Pure white needlelike crystals $C_6H_4(OH)_2$.

Hydrochloric acid: 10 per cent solution (concentrated acid diluted ten times).

Sulfuric acid: Concentrated H_2SO_4.

Sodium hydroxide: 20 or 25 per cent solution.

Ammonium hydroxide: 28 per cent (concentrated).

Reagent "M": Dissolve 1 mg. of p-nitrobenzene-azoresorcinol in 100 c.c. of normal sodium hydroxide (Eastman).

Nessler's solution: Consult textbooks for the preparation of the Bock-Benedict or Folin formula for blood analyses.

Sodium cyanide: Prepare a 5 per cent solution and preserve with 1 c.c. of 28 per cent ammonium hydroxide per 500 c.c. of solution.

Sodium nitrite: A fresh solution, 0.1 per cent, should be prepared once a week, although it often keeps much longer.

Sodium nitroprusside (nitroferricyanide): Prepare a 5 per cent solution and discard when it shows signs of fading. Protect from light.

Reagent "S": Dissolve 100 mg. of N-(1-naphthyl)ethylenediamine dihydrochloride (La Motte Chemical Products Company) in 100 c.c. of water. Store in an amber bottle.

Reagent "S" (alternative): Dilute 1 c.c. of dimethyl-*a*-naphthylamine (Eastman) in 250 c.c. of 95 per cent alcohol.

Nitric acid: Concentrated (unnecessary unless murexide supplementary test is employed).

Procedures.—The general scheme of analysis is presented in tabulated form. The calculus should be split so as to reveal different structures within, and the core or nucleus should be distinguished from the various layers by separate analysis. For full evaluation of the results the relative sensitivity of the various tests should be taken into account. The findings depend largely on the methods used to detect possible constituents of the stone. Since the calculus is constantly bathed in urine during its formation, traces of every substance found therein would be discovered in the stone if sufficiently delicate procedures were employed. The purpose of our scheme of analysis is to reveal the chief components of the stone, the minor constituents being of research rather than practical importance insofar as the present state of knowledge is concerned.

When the reagents for the Benedict-Theis method of determining serum *phosphate* are used on urinary calculi, they give a "positive" reaction with a majority of stones, including some thought to be "pure" cystine. It is, therefore, impractical to employ such a test.

The murexide test for *uric acid* is less sensitive than the recommended reagents, although it must be stressed that only a deep blue color should be re-

TABLE I

SCHEME OF ANALYSIS OF URINARY CALCULI

Chemical Group	*Reagents Added*	*Results*
1. Urates	Pulverized stone 1 drop Na_2CO_3 2 drops uric acid reagent	Prompt deep *blue* color (pale blue is negative)
2. Phosphates*	Pulverized stone 4-5 drops ammonium molybdate solution (need excess of reagent)	Warm over flame to get distinct *yellow precipitate*, $(NH_4)_3PO_4 \cdot 12\ MoO_3$
3. Oxalates	Pulverized stone Pinch of resorcinol 1 drop conc. H_2SO_4 Mix	Slow development of dull *bluish-green* color (Higgins and Mendenhall[5])
4. Oxalates	Pulverized stone 2-3 drops HCl. If no effervescence, add pinch of MnO_2. No not mix	Tiny *bubbles* of gas "explosively" released from bottom
5. Carbonates	Relatively large sample of pulverized stone 8-10 drops HCl	*Effervescence*
Take up acid extract from *5* in aspirating pipette the tip of which is lightly plugged with cotton. Remove cotton by seizing projecting wisp and divide "filtrate" into three aliquots for *6*, *7*, and *8*.		
6. Calcium*	Acid extract 2-3 drops NaOH	Fine *white* precipitate or film from oxalate stones; dense precipitate from phosphate stones
7. Magnesium	Acid extract 2-3 drops NaOH 2-3 drops reagent "M"	Reddish-purple reagent slowly becomes definitely *blue* (precipitate forms)
8. NH_4 group	Acid extract 2-3 drops NaOH 2-3 drops Nessler's solution *Alternative:* Pulverized stone 2-3 drops Nessler's solution	*Yellowish-orange* precipitate
9. Sulfonamides	Pulverized stone 2 drops HCl (wait 30 seconds) 2 drops $NaNO_2$ (wait 30-60 seconds) 2-3 drops reagent "S"	*Brownish-pink* to *magenta*
10. Sulfonamides	Pulverized stone 8-10 drops HCl Stir with broken piece of match stick or wooden applicator Let stick remain *in situ* for several minutes	Lignin in wood becomes *yellow* or *orange*. Use stick for "writing" on paper towel or newspaper. The colorless acid extract turns *yellow* on lignin-containing paper but not on rag stock (Halley[3])
11. Cystine	Pulverized stone 1 drop NH_4OH 1 drop NaCN (wait 5 minutes) 2-3 drops sodium nitroprusside	*Beet-red* color; on standing may fade to orange red

*Use a microscope slide for these tests, spot plate for the others. The artist's type is more satisfactory than the regular chemist's spot plate.

garded as "positive" (test 1). The murexide test, however, is much more useful and informative as shown later.

Nessler's solution (test 8) will detect smaller amounts of *ammonia* than will pink litmus paper when exposed to the gas released by treatment of an acidified extract of the calculus with sodium hydroxide.

The Marshall reagents will find smaller amounts of *sulfonamides* than will the lignin test of Halley. A relatively large sample of stone should be used for the latter procedure; even then the presence of an appreciable quantity of sulfonamide may not be detected.

Suitable tests for *oxalate* are not as sensitive or as specific as one might wish. In the experience of one of us (M. R. M.) the conversion of oxalate to carbonate by manganese dioxide has proved highly satisfactory (test 4). When only a minute fragment is available, oxalates can be found by using a narrow test tube and 5 to 10 drops of acid, and watching carefully with a magnifying lens for the ejection of a few bubbles of gas on addition of the black powder. This test for oxalates is not applicable to carbonate stones until all carbon dioxide has been removed from an acid extract by boiling. The solution must be cool before the manganese dioxide is added. However, we have rarely encountered carbonate in interfering amounts in oxalate stones, although Kamlet[6] reports finding carbonate in 12 out of 88 oxalate stones. With moderate-sized samples the test is easily carried out on a white or clear-glass spot plate if the black powder is not mixed after it falls upon the particles of stone. After a moment's hesitation tiny bubbles of gas are shot out like rockets. The effervescence seen after contact of hydrochloric acid with carbonate is different; the powdered stone is buoyed up by the immediate formation of many very fine bubbles of carbon dioxide. The Higgins-Mendenhall[4] test for oxalates (test 3), while specific and simple to perform, produces a poor color and that rather slowly, and requires a fairly generous test sample for easy recognition of oxalates.

Physical Characteristics.—Instances occur where the stone is minute or only a small part of it is available for study, necessitating the use of a few selected tests dependent on the examiner's ingenuity. Fortunately, the appearance of a stone generally gives a clue to its composition.

Uric acid often occurs as multiple, smooth, round pebbles, which are devoid of luster and vary in size from a few millimeters to 30 mm. or more in diameter. Single stones frequently have a bumpy or eruptive type of surface, resembling miniature volcanic craters. Uric acid stones are always definitely yellow, but this color may not be noted until the stone is crushed.

Phosphate and *carbonate* stones occur as compact balls (often several centimeters in diameter) or as large friable masses assuming the shape of the cavity were found; these are clay or chalk-like. Rarely, triple phosphate stones ($MgNH_4PO_4$) assume porous, coral-like formations, which suggest calcium oxalate except for their whiteness.

Oxalate stones show considerable variety in form. Irregular, light-colored fragments may exhibit elaborate crystalline structures with sharp projecting blades. Oxalate stones are conspicuous for their crystalline glint or smooth luster. The dark-brown type is very compact and may take a grape-seed or berry-like form, which should be differentiated from the prostatic corpora amylacea. If the rounded contours are larger, like a bunch of grapes, the calculi may be described as botryoidal. Although the berry and compact stratified forms are dark brown, crushing produces a light-colored powder. Mammillated

oxalate stones occur, although the nipplelike structure is rarer than a prickly surface, resembling the "eruptive" type of uric acid calculus. Buff-colored or brown stones may be covered with a fine coral-like excrescence. Occasionally, the whole structure is loose and porous.

Cystine stones occur largely as pale yellow or white granules upon which the pestle seems to skid as it crushes the calculus. Multiple stones may develop in the form of uric acid pebbles, except that they are usually much smaller, many hardly reaching 1 mm. in diameter. Such stones possess a faint waxy luster and are brownish in color which distinguishes them from the yellow uric acid pebbles. Another form of cystine stone resembles calcium oxalate on casual inspection since it presents a crystalline surface.

The *sulfonamides* may be deposited on the surface of uric acid without obvious layering or may be mingled with phosphate and carbonate in a putty-like mass. There is nothing in the physical appearance of the stone to suggest the presence of sulfonamides. It is yet to be determined whether or not "pure" sulfonamide calculi will occur.

It is important to analyze prominent layers of calculi when possible. Although uric acid stones often are "pure," they frequently pick up a thick crust or coating of different nature, usually phosphates unless sulfonamides are available. Phosphates do not, as a rule, deposit as a visible crust on oxalate stones but occur throughout the calculus. Instances are seen where uric acid encircles an oxalate nucleus several millimeters in diameter and then is thickly encased in magnesium ammonium phosphate and calcium carbonate.

Supplementary Tests.—Tests other than those described in the table may be desired by the technician for guidance or for checking purposes. The following tests are valuable in that they furnish a lead as to the presence of substances other than those sought.

Murexide Test.—When a sample of pulverized stone is treated with several drops of concentrated nitric acid and carefully evaporated to dryness, then subjected to 2-3 drops of ammonia water, the following may be observed:

Uric acid— deep yellow to orange-red or crimson becoming more purplish with ammonium hydroxide (bluish violet with sodium hydroxide).

Xanthine— greenish-yellow turning to orange with ammonium hydroxide becoming reddish on warming (deep orange-red with sodium hydroxide).

Protein— pale yellow turning to orange.

Sulfonamides—yellow turning to mahogany brown.

It should be emphasized that the murexide test for uric acid usually is carried out in an evaporating dish on a water bath, but this may not provide a sufficiently high temperature for the xanthine reaction. It is very easy to miss xanthine in urinary calculi.

According to commonly used "systems" of stone analysis, no gas is evolved when concentrated nitric acid is added to a pulverized uric acid calculus, whereas effervescence occurs in the presence of ammonium urate. Domanski[1] emphasizes this point and further states that uric acid is not appreciably soluble in nitric acid, although urates dissolve readily. In our experience both uric acid and urates are soluble under these conditions. Uric acid, whether reagent pure, re-

covered from a lateritious deposit or obtained in calcareous form, reacts with concentrated nitric acid so as to release a continuous stream of bubbles until the powder is no longer visible. In the case of stones, the effervescence is quite frothy whereas it is quieter and confined to a swirling motion within the fluid when pure uric acid is tested, but this may be due solely to the fact that the reagent grade acid exists in a more finely divided form than does the crudely ground calculus. All stones giving a positive murexide test have shown this gas formation whether ammonium urate was present or not.

In this connection a further point needs clarification. The standard method for testing for the ammonium radical in urinary stones involves the addition of strong alkali for the release of ammonia gas and its detection by wet pink litmus paper or filter paper treated with Nessler's solution. Domanski found that ammonia was not obtained from ammonium urate by this process. We have had no difficulty in distinguishing between ammonium urate and uric acid stones by the method described in this paper (test 8). However, ammonium urate stones appear to be relatively rare—which may be less fact than failure to discover the ammonium radical.

Ashing Process.—Another helpful procedure is to ash a sample of the stone in a porcelain crucible. This is a desirable preliminary to the test for phosphates given in the table (test 2) but is not essential. It is difficult to evaluate the odor of the heated stone except in the case of cystine. This penetrating odor, once experienced, is never forgotten. It follows the first curl of smoke and diminishes in intensity with increased heat. Both cystine and urate stones blacken rapidly, the former burning "clean" and the latter with advancing greasy brown rings up the crucible; both leave the crucible empty. The residue from phosphate and oxalate stones is of about the same bulk as the original sample. The phosphates rarely give a white ash even after long and hard heating. With oxalates the ash is more nearly white and effervesces if acid is added as in testing for phosphorus since oxalate is converted into carbonate by heating. This effervescence is not to be confused with spattering obtained on adding a fluid to the hot ash. Sulfonamides melt and form a black tarry mass which is resistant to ashing. It can be seen, then, that the response to heating may furnish valuable clues as to the composition of a calculus.

SUMMARY

1. A scheme of urolith analysis is presented which is simple, rapid, and dependable.

2. A number of calculi or separate layers can be analyzed simultaneously with the simple equipment required.

3. The physical characteristics of urinary stones and supplementary means for testing their composition are discussed.

Appreciation is expressed to Dr. Joseph A. Hyams for his stimulating interest in this investigation.

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A CRITICAL STUDY OF THE CARR-PRICE REACTION FOR THE DETERMINATION OF β-CAROTENE AND VITAMIN A IN BIOLOGICAL MATERIALS*

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SEVERAL methods based on the Carr-Price[1] reaction for the determination of β-carotene and vitamin A in biological materials have been described and widely applied to nutritional studies.[3, 7-9, 11, 13] The limitations of the reaction have been pointed out on several occasions[3, 7] and summarized recently[12] with the conclusion that under properly controlled conditions the antimony trichloride reaction with β-carotene and vitamin A is suitable for some studies, although spectrophotometric and biological assays give more reliable results. Sakamoto[14] found good agreement between values for β-carotene and vitamin A obtained by polarimetric and colorimetric methods. Embree[4] finds that the measurement of the color produced by antimony trichloride in the presence of vitamin A by the use of the photoelectric colorimeter is almost as exact as that given by the spectrophotometer. Salter,[15] on the other hand, in a recent review stated that the methods for vitamin A based on the Carr-Price reaction are too erratic for routine use.

We have had occasion in this laboratory to use the Carr-Price reaction for the analysis of a large number of samples of blood plasma or serum, vitamin concentrates, and rat tissues. In view of the recent criticism of the reaction, it appeared important to establish the validity of the results obtained by such a procedure. Therefore, the present work was undertaken.

EXPERIMENTAL

Two modifications of the Carr-Price reaction, both of which involve the use of the Evelyn colorimeter, have been studied. One is the micromethod described in detail by May, Blackfan, McCreary, and Allen,[10] and the other is

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the macroprocedure outlined briefly by Lewis, Bodansky, and Haig.[9] The two methods were compared for the reproducibility of results on different and on the same samples of blood plasma. Since the macroprocedure was found satisfactory in our hands, we shall describe it in detail. Recovery studies of crystalline β-carotene and vitamin A employing the macroprocedure were also made. Although Embree[4] found that serious loss of vitamin A potency results from exposure of solutions of vitamin A in clear glass test tubes to ultraviolet light for four to five and a half hours, we used throughout only clear glass glassware. Since the average time necessary to complete the determinations was an hour or less, the excellent recoveries obtained indicate that the destructive influence of ultraviolet light under the conditions used was not in evidence. However, when a large number of samples are run at the same time, requiring several hours for the completion of the determinations, Embree's[4] suggestion to use amber glassware to prevent the destruction of vitamin A by ultraviolet light becomes of practical importance, particularly in the case of samples of low vitamin A potency.

MACROPROCEDURE. BLOOD PLASMA OR SERUM

Five milliliters of 95 per cent alcohol and 8 ml. of petroleum ether (B.P. 35° to 75° C.) are added to 3 to 5 ml. samples of blood plasma or serum, and the tubes are shaken on a mechanical shaker for ten minutes. After centrifuging two or three minutes at 1,300 r.p.m., 6 ml. of the petroleum ether layer are transferred to a previously calibrated colorimeter tube, and the carotene reading is taken with filter 440. The Evelyn tube is immersed in a water bath at about 65° C. and is connected to a water pump. Evaporation is complete in four or five minutes. Prolonging the time of evaporation to ten or fifteen minutes was found to lower the vitamin A values by about 10 per cent. The residue is dissolved in 0.6 ml. of chloroform, and with the tube inserted in the colorimeter, 5.4 ml. of 25 per cent antimony chloride in chloroform are added as quickly as possible. The minimum galvanometer reading with filter 620 is taken within three seconds before the color developed begins to fade.

The carotene values are calculated with the constants of Koehn and Sherman,[7] and for vitamin A we have used the formula of Dann and Evelyn[3] and the factor of 2,100 given by Holmes and Corbet[5] for converting $E\ \frac{1\%}{1\ cm.}$ (328 mμ) to international units.

$$\frac{L_{440} \times 2.65 \times 6 \times 100}{\frac{3}{4} \times \text{ml. of sample}} = \text{Micrograms of carotene per 100 ml.}$$

$$\left[\frac{L_{620}}{\frac{3}{4} \times \text{ml. of sample}} - \frac{0.14\ L_{440}}{\frac{3}{4} \times \text{ml. of sample}}\right] \times 52.5 \times \frac{6}{100} \times 0.41 \times 2{,}100 = \text{I. U. vitamin A per 100 ml.}$$

RAT LIVER

One gram samples of liver are heated with 5 ml. of saturated potassium hydroxide in 95 per cent alcohol at 75°-80° C. for forty-five minutes with occasional shaking. After cooling, the digests are made up to 10 ml. with water.

For liver samples containing about 100 to 1,000 international units per gram; an aliquot of 0.2 ml. is taken for analysis. The sample is diluted to about 5 ml. with water and from this point the determination is carried out in the same way as was described for blood plasma.

Applying this procedure to the livers of rats maintained on diets of various nutritional qualities, we obtained concordant results with satisfactory reproducibility.[14]

DISCUSSION

Table I shows data obtained by the microprocedure of May, Blackfan, McCreary, and Allen[10] and the procedure described above. The objective was to check the reproducibility of the values obtained by both procedures on different samples of blood plasma. Table II illustrates the analytical values obtained by both procedures on the same samples. It will be seen from the data presented that the macroprocedure described above gives consistently better and more reproducible values than the microprocedure. We believe that this difference in the performance of the procedures is not due to any dif-

TABLE I

REPRODUCIBILITY OF RESULTS FOR β-CAROTENE AND VITAMIN A IN HUMAN BLOOD PLASMA BY THE MICROPROCEDURE AND MACROPROCEDURE

	SAMPLE I		SAMPLE II	
	MICROMETHOD		MACROMETHOD	
	CAROTENE μG/100 ML.	VITAMIN A I.U./100 ML.	CAROTENE μG/100 ML.	VITAMIN A I.U./100 ML.
	128.9	----	116.1	148.0
	105.8	100.3	121.8	152.1
	141.0	62.6	120.7	153.9
	119.9	79.7	121.8	152.1
	122.9	62.9	118.4	151.0
	116.9	77.1	125.1	168.4
	134.9	64.1	118.4	154.4
	119.9	67.6		
Average	123.8	73.5	120.3	154.3
Maximum deviation from mean	14.5%	36.5%	4.0%	9.1%
Average deviation from mean	6.8%	14.3%	1.9%	2.6%

TABLE II

COMPARISON OF VALUES FOR β-CAROTENE AND VITAMIN A OBTAINED ON THE SAME SAMPLE OF BLOOD PLASMA BY THE MICROPROCEDURE AND MACROPROCEDURE

SAMPLE	MACROMETHOD		MICROMETHOD	
	CAROTENE μG/100 ML.	VITAMIN A I.U./100 ML.	CAROTENE μG/100 ML.	VITAMIN A I.U./100 ML.
1	168.6	258.2	144.0	227.5
	173.9	318.9	119.9	222.7
	171.3	301.2	138.0	208.0
Average	171.3	292.8	134.0	219.4
2	171.3	224.2	138.0	147.8
	172.6	241.6	----	----
Average	172.0	232.9	138.0	147.8
3	63.7	40.0	43.3	24.1
	63.7	42.9	46.3	----
Average	63.7	41.5	44.8	24.1

ference in the basic principle employed, but to the difficulty in carrying out all the steps involved in a sufficiently short time by the microprocedure as is essential to the accuracy of the reaction.

Table III indicates that no significant increase in the vitamin A resulted after a preliminary saponification of 3 ml. samples of plasma in alcoholic potassium hydroxide for twenty minutes. It appears that most of the vitamin in normal human plasma exists in the free form, as has been suggested previously.[2]

TABLE III

EFFECT OF SAPONIFICATION ON THE β-CAROTENE AND VITAMIN A VALUES OF NORMAL HUMAN BLOOD PLASMA

SAMPLE	CAROTENE μG/100 ML.		VITAMIN A I.U./100 ML.	
	UNSAPONIFIED	SAPONIFIED	UNSAPONIFIED	SAPONIFIED
1	243.2	241.9	130.6	136.5
2	215.8	206.8	131.5	141.1
3	98.8	88.2	153.8	154.6
4	160.8	151.8	128.2	134.0
5	146.8	145.6	107.1	100.4
6	110.6	106.7	127.6	137.6
7	307.7	285.0	122.8	132.0
8	103.1	103.1	119.1	120.7
Average	173.4	166.1	127.6	132.1

TABLE IV

RECOVERY OF CRYSTALLINE β-CAROTENE AND VITAMIN A FROM PETROLEUM ETHER

CAROTENE		VITAMIN A		
ADDED	FOUND	ADDED		FOUND
μG	μG	μG	I. U.*	I. U.
0.464	0.484	0.463	1.389	1.470
0.696	0.748	0.695	2.085	1.944
0.928	1.003	0.926	2.778	2.628
1.160	1.265	1.158	3.474	2.700
2.320	2.389	2.316	6.948	6.840
3.480	3.498	3.474	10.422	10.980
4.640	4.650	4.632	13.896	13.560
5.800	5.995	5.790	17.370	18.225
6.960	7.059	6.948	20.844	19.710
8.120	8.203			
9.280	9.240			
10.440	10.380			
11.600	11.290			
12.760	12.240			
13.920	13.450			

*The weights of crystalline vitamin taken were converted to international units by means of the relationship of 3 I. U. per microgram given by Holmes and Corbet.[5]

Recoveries of carotene and vitamin A were made on separate solutions of known concentration. The average deviation from the theoretical values, as calculated from the figures above, was +1.6 per cent for carotene and −1.9 per cent for vitamin A.

Tables IV and V show data illustrating the recovery of crystalline β-carotene and vitamin A from petroleum ether, either separately or when mixed in various proportions. As will be seen, the procedure outlined above gives excellent recoveries with reasonable experimental errors. In Table VI are presented data for the recovery of β-carotene and vitamin A from normal human plasma. Again the recoveries were sufficiently good to warrant the use of the procedure for routine use.

TABLE V

RECOVERY OF MIXTURES OF CRYSTALLINE β-CAROTENE AND VITAMIN A FROM PETROLEUM ETHER

SERIES	CAROTENE		VITAMIN A		
	ADDED	FOUND	ADDED		FOUND
	μG	μG	μG	I. U.*	I. U.
I	0.708	0.728	0.421	1.263	1.434
	2.123	2.103	0.421	1.263	1.341
	3.540	3.557	0.421	1.263	1.170
	4.957	4.692	0.421	1.263	1.218
	6.490	6.201	0.421	1.263	1.110
II	0.708	0.803	3.507	10.521	10.680
	2.123	2.103	3.507	10.521	10.377
	3.540	3.499	3.507	10.521	10.560
	4.957	4.723	3.507	10.521	10.347
	6.490	6.237	3.507	10.521	10.635

*The weights of crystalline vitamin taken were converted to international units by means of the relationship of 3 I. U. per microgram given by Holmes and Corbet.[5]

The average deviation from the theoretical values in series I was −0.2 per cent for carotene and +2.2 per cent for vitamin A; in series II, for carotene it was +10.8 per cent and for vitamin A −0.6 per cent.

TABLE VI

RECOVERY OF CRYSTALLINE β-CAROTENE AND VITAMIN A ADDED TO BLOOD PLASMA

PLASMA LEVELS		CAROTENE		VITAMIN A*		TOTAL RECOVERED	
CAROTENE	VITAMIN A	ADDED	TOTAL	ADDED	TOTAL	CAROTENE	VITAMIN A*
μG/100	I. U./100	μG/100	μG/100	I. U./100	I.U./100	μG/100	I. U./100
ML.	ML.	ML.	ML.	ML.	ML.	ML.	ML.
120.7	167.5	216.3	337.0	56.1	223.6	335.9	223.6
120.7	167.5	23.6	144.3	280.5	448.0	144.4	451.1
120.7	167.5	118.0	238.7	168.3	335.8	233.2	333.9

*The weights of crystalline vitamin taken were converted to international units by means of the relationship of 3 I. U. per microgram given by Holmes and Corbet.[5]

Petroleum ether solutions of β-carotene and vitamin A of known concentration were added to normal human blood plasma in which the concentrations of carotene and vitamin A were previously determined.

SUMMARY

The applicability of the Carr-Price reaction to the analysis of biological materials has been critically re-examined using the Evelyn photoelectric colorimeter. Excellent recoveries of crystalline β-carotene and vitamin A were obtained with reproducible results. The macroprocedure described was found satisfactory for routine analysis of blood plasma or serum. Preliminary saponification of plasma or serum was found to have no significant effect on the vitamin A or carotene concentrations in normal human plasma. Recent criticisms directed against the Carr-Price reaction as the basis for the quantitative determination of vitamin A and β-carotene in biological materials should take into account the carefully specified conditions which are essential to the successful application of this reaction.

The studies referred to herein were conducted under the auspices of the International Health Division of the Rockefeller Foundation of New York City by the nutrition units of the Departments of Biochemistry and Medicine, Vanderbilt University School of Medicine, Nashville, Tenn.

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PROTHROMBIN STUDIES USING RUSSELL VIPER VENOM

IV. Relation Between Prothrombin Clotting Time and Plasma Fibrinogen Concentration

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THE clinical interpretation of laboratory tests must be based on a knowledge of factors that may cause a variation in the test. The increasing use of the prothrombin clotting time test in studying bleeding and hemorrhage brings up the question of the relation of prothrombin clotting time to the blood concentration of other factors involved in the coagulation mechanism (calcium, thromboplastin, fibrinogen, etc.).

The study reported here consisted of obtaining blood from healthy adults and from patients with chronic ulcerative colitis or miscellaneous gastrointestinal diseases and determining the prothrombin clotting time and the fibrinogen content of the plasma.

METHOD OF STUDY

Blood of healthy normal adults and of patients attending an outpatient clinic was obtained. Most of the blood was obtained in the afternoon so these were mainly postprandial specimens. Blood for determination of the prothrombin clotting time and fibrinogen content was drawn at the same time.

The method employed was Quick's procedure[1] modified by using Russell viper venom instead of brain thromboplastin.[2-4] Four and one-half cubic centimeters of venous blood were drawn into a dry syringe and mixed with 10 mg. of potassium oxalate in a centrifuge tube. The blood was centrifuged at 1,500 r.p.m. for five minutes, and the oxalated plasma was drawn off. The test was performed within two hours after withdrawal of the blood; if hemolysis was present, the sample was discarded. The test was performed as follows: 0.2 c.c. of oxalated plasma was pipetted into a small test tube (75 by 10 mm.), and 0.2 c.c. of Russell viper venom,* 1:10,000 solution, was added. Calcium chloride solution (1.11 Gm. calcium chloride per 100 c.c.), 0.2 c.c., was then added, and the stop watch immediately started. The tube was agitated for from ten to fifteen seconds and tilted until separate discrete fibrin particles could be seen. The prothrombin clotting time in normal individuals using Russell viper venom has been found to be 20.76 seconds ± 2.32 seconds[3] and 19.5 seconds ± 2.9 seconds.[5]

The method used for the fibrinogen content of blood plasma was a combination of the methods of Howe[6] and Wu[7] and Koch and McMeekin[8] as

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*Supplied as "Stypven" Russell Viper Venom by The Burroughs Wellcome & Co. (U. S. A.), Inc., New York, N. Y.

adopted in the Biochemical Laboratories of the Philadelphia General Hospital. One cubic centimeter of oxalated plasma was diluted with 48 c.c. of physiologic saline. One cubic centimeter of calcium chloride was then added and the mixture was allowed to stand for thirty minutes. The clot was removed, dried, and placed in an ignition tube with 1 c.c. of acid digestion mixture. Five drops of caprylic alcohol were added to prevent foaming. The mixture was boiled vigorously and when white fumes appeared, superoxol (30 per cent H_2O_2) was added to clear the solution (about 0.5 c.c.). The solution was again heated vigorously and then cooled. Distilled water was added to make a total volume of 35 c.c. Fifteen cubic centimeters of Nessler's solution were then added, the solution was centrifuged, and the supernatant fluid was matched against standards containing from 0.6 to 1.0 mg. of nitrogen. The normal range for plasma fibrinogen with this procedure is 0.2 to 0.4 Gm. per 100 c.c.

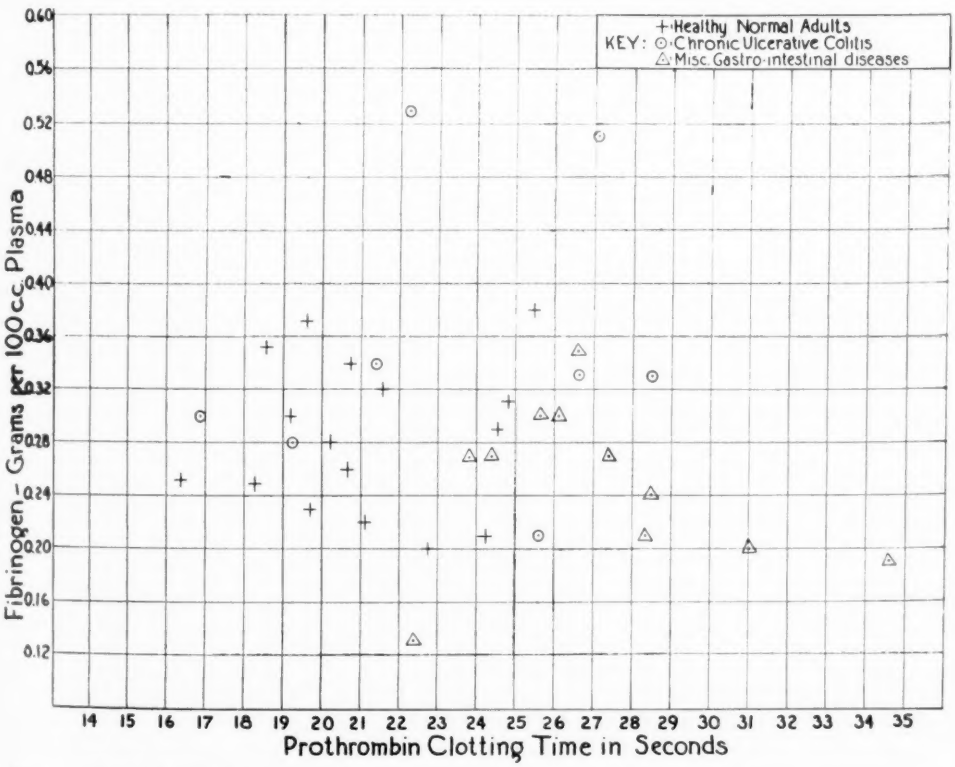

Fig. 1.—Chart showing relation between prothrombin clotting time and plasma fibrinogen in normal adults and in patients with gastrointestinal disorders.

DISCUSSION OF RESULTS

The results are plotted in Fig. 1. It will be observed that the points are widely scattered, and there is little evidence of any trend except possibly that longer prothrombin clotting times are associated with lower concentrations of fibrinogen. This possibility was investigated by calculating the correlation coefficient which was found to be –0.145. Since this figure fails to exceed the tabular value of 0.3246 given by Fisher and Yates[9] for a probability of 0.05

which corresponds to 1 chance in 20, no correlation can be said to exist. Consequently, the prothrombin time in these data is independent of the amount of fibrinogen in the plasma. However, this does not exclude the possibility that in certain as yet unknown diseases a correlation between these two variables will be found.

SUMMARY AND CONCLUSIONS

No relationship was found to exist between the prothrombin clotting time and the concentration of fibrinogen in the plasma in normal subjects and in patients with a variety of gastrointestinal lesions.

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PROTHROMBIN STUDIES USING RUSSELL VIPER VENOM

V. Interbatch Variations of Venom

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RUSSELL viper venom has been used for more than two years by us as a thromboplastic agent in a modified form of Quick's prothrombin test.[1] The modification consists in substituting Russell viper venom for brain thromboplastin. However, more information on the stability and uniformity of different batches of Russell viper venom is desirable when it is to be used in such a quantitative time relation test.

Other workers using Russell viper venom in this test have mentioned the subject of interbatch variations. Witts and Hobson[2] stated that different

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batches of Russell viper venom as purchased vary in coagulant power but that vials from the same batch are very constant. Crosbie and Scarborough[3] used two different batches of venom which did not differ appreciably in activity.

The object of the present study was twofold: (1) to learn how much variation existed between batches of Russell viper venom and (2) to determine if ageing had any effect on the potency of this type of preparation.

DESCRIPTION OF PREPARATION

The venom of the Russell viper (*Vipera russellii*) is collected by the usual methods employed at snake farms in India. The venom is then dried, sterilized, and standardized by comparing it with a sample of known potency on fowl and horse plasma. Following this the venom is packed in dry form in nitrogen-filled glass ampoules.

Venom in ampoules, of different batches, has been collected for the past four years and kept at room temperature so that when this study was begun samples from thirteen different batches were available and they varied in actual age up to five and one-half years.*

METHOD OF STUDY

Four essentially normal adults were selected, venous blood samples were obtained and prothrombin clotting times determined for each blood sample with each of the thirteen batches of venom.

The method employed was Quick's procedure[1] modified by using Russell viper venom instead of brain thromboplastin.[4, 5] Eighteen cubic centimeters of venous blood were drawn into a dry syringe and mixed with 40 mg. of potassium oxalate in a centrifuge tube. The blood was centrifuged at 1,500 r.p.m. for five minutes, the oxalated plasma was drawn off, and the tests were done immediately. The test on each batch was performed as follows: 0.2 c.c. of oxalated plasma was pipetted into a small test tube (75 by 10 mm.) and 0.2 c.c. of Russell viper venom,† 1:10,000, solution was added. Calcium chloride solution (1.11 Gm. calcium chloride per 100 c.c.), 0.2 c.c., was then added and the stop watch immediately started. The tube was agitated for from ten to fifteen seconds and tilted until separate discrete fibrin particles could be seen. The prothrombin clotting time in normal individuals using Russell viper venom has been found to be 20.76 seconds ± 2.32 seconds[5] and 19.5 seconds ± 2.9 seconds.[6] Each of the four subjects had essentially a normal prothrombin clotting time.

RESULTS OF STUDY

The results are given in Table I. In order to determine whether significant variations existed between batches and whether there was evidence of deterioration with age, the data were plotted as in Fig. 1. It will be observed that the mean prothrombin clotting times obtained with all batches regardless of age fell

*In the experiments, age of venom refers to elapsed time in days between the labeled "expiration date" and the date of the experiment. The venom actually was one year older than these figures indicate because the expiration date on the ampoule is one year in advance of the date on which the venom was placed in the ampoule.

†Supplied as "Stypven" Russell Viper Venom by The Burroughs Wellcome & Co. (U. S. A.) Inc., New York, N. Y.

TABLE I

DATA ON PROTHROMBIN CLOTTING TIMES OF FOUR ADULTS AS OBTAINED WITH VENOM OF THIRTEEN DIFFERENT BATCHES

VENOM BATCH NO.	AGE* IN DAYS	PROTHROMBIN CLOTTING TIME (SEC.)				MEAN BATCH PROTHROMBIN CLOTTING TIME (SEC.)
		PATIENT A	PATIENT B	PATIENT C	PATIENT D	
RV 30 C	1,632	20.0	19.5	23.2	23.7	21.6
RV 99 B	1,046	21.2	20.2	30.0	25.5	24.2
RV 122 D	981	22.0	20.8	24.0	23.8	22.6
RV 134 A	881	23.8	21.0	23.4	24.2	23.2
RV 146 A	731	21.7	21.6	27.5	21.2	23.0
RV 158 B	610	22.6	20.4	28.4	24.8	24.0
RV 164 A	566	23.5	21.7	23.0	24.0	23.0
RV 191 B	276	22.8	21.9	28.1	23.0	23.9
RV 199 D	177	23.8	23.1	28.4	24.2	24.8
RV 200 A	171	20.8	19.8	25.8	22.4	22.2
RV 210 A	37	23.8	21.1	21.7	21.9	22.1
RV 212 A	27	22.8	20.1	23.0	24.1	22.5
RV 230 B	-224	22.8	20.0	23.4	24.0	22.3

*See first footnote on p. 913.

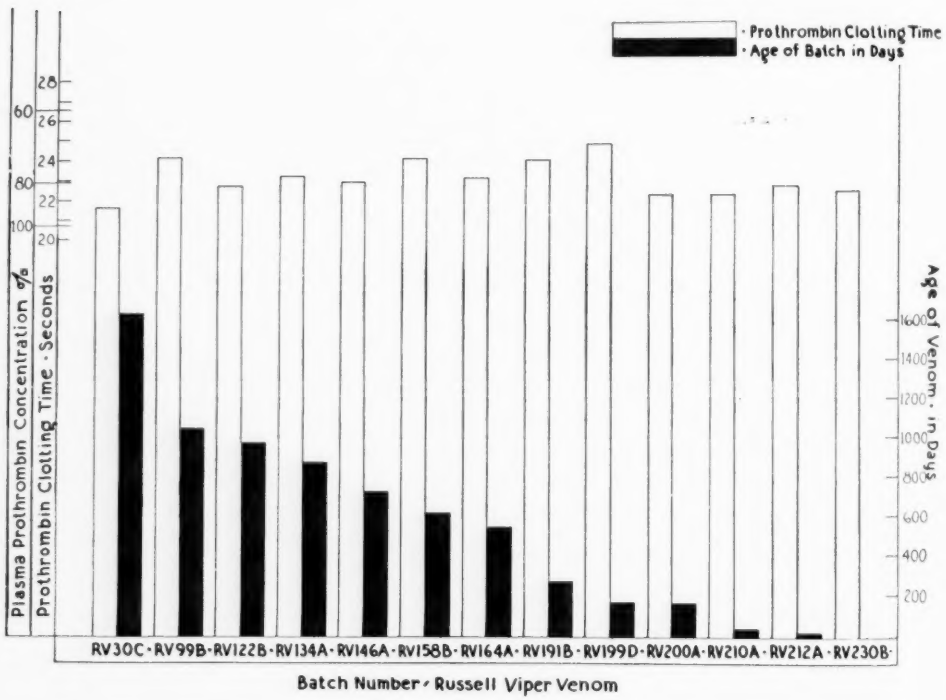
Prothrombin Clotting Time
Age of Batch in Days
Plasma Prothrombin Concentration %
Prothrombin Clotting Time - Seconds
Age of Venom - in Days
RV30C · RV99B · RV122B · RV134A · RV146A · RV158B · RV164A · RV191B · RV199D · RV200A · RV210A · RV212A · RV230B
Batch Number - Russell Viper Venom

Fig. 1.—Bar graph showing the variations in prothrombin clotting time obtained with thirteen batches of Russell viper venom of different ages. Clear columns represent mean prothrombin clotting time; black columns represent age in days (time elapsed between expiration date and day of experiment) of the particular batch of venom.

within very narrow limits, roughly twenty-two to twenty-four seconds. Also, although the age of the oldest batch was 1,632 days (almost four and one-half years), its mean prothrombin time value (21.6 seconds) was almost the same as that obtained with the newest batch bearing an expiration date that would

not be reached until about eight months after this study and having a mean prothrombin clotting time of 22.3 seconds. It seems obvious, therefore, that as far as this study goes, age did not affect the potency of the preparation.

A statistical examination of the data, in which an analysis of variance was calculated, revealed that no significant variation between batches existed. F, the variance ratio, was 1.386 which, according to Snedecor's tables,[7] is not to be considered significant. If patients with prolonged prothrombin clotting times had been used in this study, greater variations would have been introduced, and the difficulty of detecting possible interbatch variations would have been increased.

SUMMARY AND CONCLUSIONS

1. Samples of Russell viper venom in nitrogen-filled ampoules were found to be stable over a period of at least five and one-half years.

2. Variations between thirteen different batches were not found to be significant.

We wish to express our appreciation for the assistance and cooperation of Dr. Louis Bauman in whose laboratory this work was carried out.

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A SIMPLIFIED TECHNIQUE FOR MEASURING RENAL BLOOD FLOW AND TUBULAR EXCRETORY MASS

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A RELIABLE simplification of Smith's original technique[1] for determining renal blood flow and tubular excretory mass would be highly desirable. We have previously reported[2] that the subcutaneous injection of diodrast and inulin can produce plasma concentrations suitable for clearance studies and that this method is very much more simple than that which depends upon constant intravenous infusion. Plasma iodine concentrations sufficiently high for the measurement of diodrast-Tm cannot be established in this way, however, so we have sought an empirical substitute. This report concerns an attempt to predict diodrast-Tm from the total quantity of diodrast excreted during the thirty minutes following a single intravenous injection of a standard dose. It appears that the percentage of diodrast excreted under these conditions is sufficiently proportional to diodrast-Tm to permit prediction of the latter from a line chart. The combination of these two modifications greatly simplifies the entire procedure and reduces the required number of blood and urine specimens by nearly one-half.

Plasma Diodrast and Inulin Clearance.—For subcutaneous use the 35 per cent diodrast solution* is irritating unless diluted about three times with saline. Inulin (Pfannstiehl, C. P.) must be thoroughly purified by boiling in water, filtering, and reprecipitating with alcohol three or four times until there is no visible residue on the filter paper; before each experiment the required amount is boiled again in 0.5 per cent saline sufficient to make a 25 per cent solution and injected while warm enough to remain in solution.

The optimal plasma concentration of diodrast is 1 to 5 mg. per 100 c.c.; of inulin, 5 to 15 mg. per 100 c.c. We originally reported that these may be achieved by the use of 0.2 c.c. diodrast and 0.2 Gm. of inulin per kilogram of body weight.[2] Extended experience has shown that amounts half this large are usually adequate, particularly in abnormal subjects. This means that a 70 kg. subject requires the subcutaneous injection of about 20 c.c. of diluted diodrast and 28 c.c. of 25 per cent inulin, but little discomfort arises since half of each solution is injected into the loose tissue of each axilla. Furthermore the injections of diodrast and inulin are separated by an interval of about forty-five minutes.

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*Supplied by The Winthrop Chemical Co.

Diodrast-iodine was determined by the method of White and Rolf[3] and inulin by a slight modification of that of Corcoran and Page.[2]

Diodrast-Tm Substitute.—In a study concerned with intravenous pyelography,[4] we have shown that normal subjects who received an intravenous injection of diodrast (0.2 to 0.6 c.c. kg. at a uniform rate during a five-minute period) excrete a relatively constant fraction of this dose during the next half hour but that the plasma iodine concentration falls somewhat below that required for Tm determination at the end of this time. Since at high plasma iodine concentrations the glomerular and tubular contributions to total urinary diodrast are approximately equal and since our abnormal subjects[5] have shown no marked disproportion between glomerular filtration rate and tubular excretory mass, it seemed likely that reductions in diodrast-Tm would be reflected by proportionate reductions in total diodrast excretion rates.

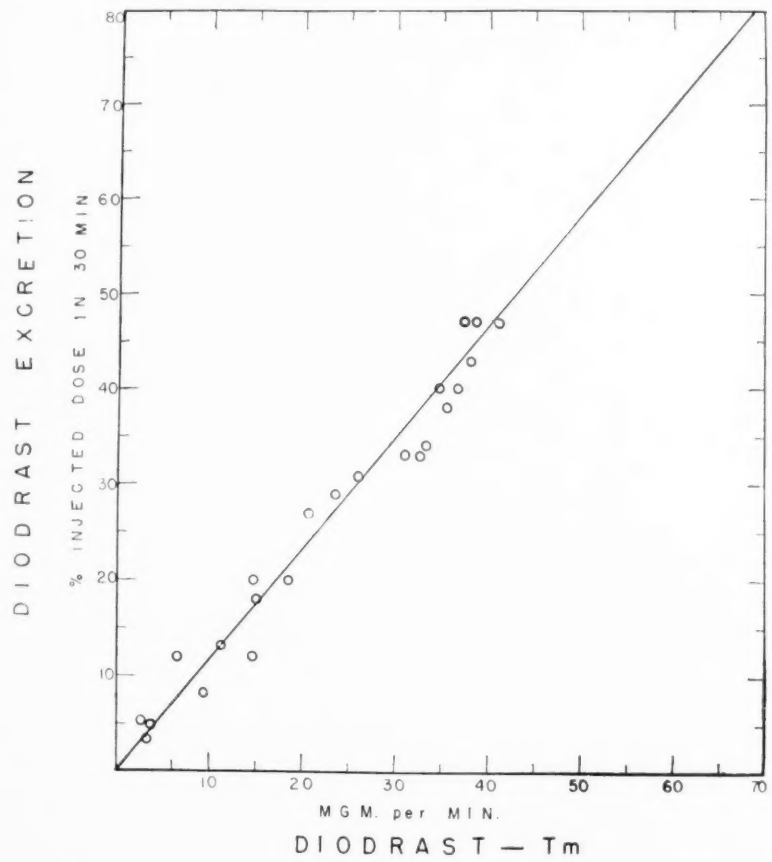

Chart 1.—Correlation between diodrast-Tm and the thirty-minute test.

Chart 1 shows results obtained on a series of normal subjects and patients with various renal disorders. The diodrast-Tm determinations were made by a slight modification of Smith's technique; most of them have been reported elsewhere.[5] Each point represents the average of at least three periods. Two or three days later each subject was given an intravenous injection of diodrast

(0.3 c.c. kg.) at a uniform rate during a five-minute period and the total amount excreted in the thirty minutes following completion of the injection determined. Expressed as a fraction of the injection dose, this value was plotted against diodrast-Tm. In Chart 1 it is not intended to minimize the occasional wide discrepancy at low Tm levels; the percentile difference is sometimes large but not, in our experience, larger than the fluctuations which have appeared in consecutive Tm periods obtained by the conventional infusion technique. This series is too small for statistical analysis, but it suggests that the error in predicting Tm from this line chart is less than ± 10 per cent.

Except for a few specially trained subjects, all of the half-hour urine collections were made by catheter and repeated bladder washings.

Combined Technique.—An indwelling catheter is applied to the fasting but hydrated subject, and a blood sample is obtained which serves as a blank for the diodrast and inulin determinations.

The subject lies on his back with his hands under his head. Under 1 per cent procaine anesthesia half of the inulin solution is injected into the subcutaneous tissues of each axilla at about the fifth interspace in the anterior axillary line. A 25 c.c. glass syringe is usually adequate and a three-inch 18-gauge needle is satisfactory. If the needle is fully inserted and slowly withdrawn during injection, no great rupture of tissue occurs. We have not massaged the areas after injection.

About forty-five minutes later the diluted diodrast is injected into the same places. This may cause momentary burning.

About fifteen minutes later the first blood sample (heparin) is obtained, the bladder is washed with saline, and clearance periods are established in the usual manner. Satisfactory plasma concentrations of diodrast and inulin exist for at least three hours.

The final blood sample is withdrawn after the last urine collection has been made. The quantity of undiluted diodrast necessary for Tm estimation (0.3 c.c. kg. in five minutes) is injected through the same needle and the bladder washed for the last time exactly thirty minutes after the end of the injection period. This sample is analyzed for diodrast only and completely replaces the minimum of three blood and three urine samples required by the intravenous technique.

It is of course essential that the catheter be draining freely and that the bladder be thoroughly washed. We have usually used five 20 c.c. portions of saline for this purpose, and all of these are added to the voided specimen before analysis.

One operator can easily examine two subjects simultaneously in this way.

DISCUSSION

Account must of course be taken of the diodrast contributed to the urine by the subcutaneous depots during the Tm period. The points in Chart 1 were obtained on diodrast-free subjects, but the combined technique described above requires that the thirty-minute test be performed on individuals whose

urine already contains diodrast coming from the axillary reservoirs. Failure to correct for the iodine from subcutaneous sources may increase the Tm estimate by 20 per cent.

Chart 2 presents the necessary correction factors; it is compiled from 195 plasma diodrast clearance periods in sixty-six subjects. Since the rate of diodrast excretion depends upon (a) the plasma concentration and (b) the plasma clearance, we have divided our renal blood flow data into five groups, depending upon the mean plasma diodrast concentration obtained during the clearance periods: 0 to 1, 1 to 2, 2 to 3, 3 to 4, and 4 to 5 mg. per 100 c.c. Each group is

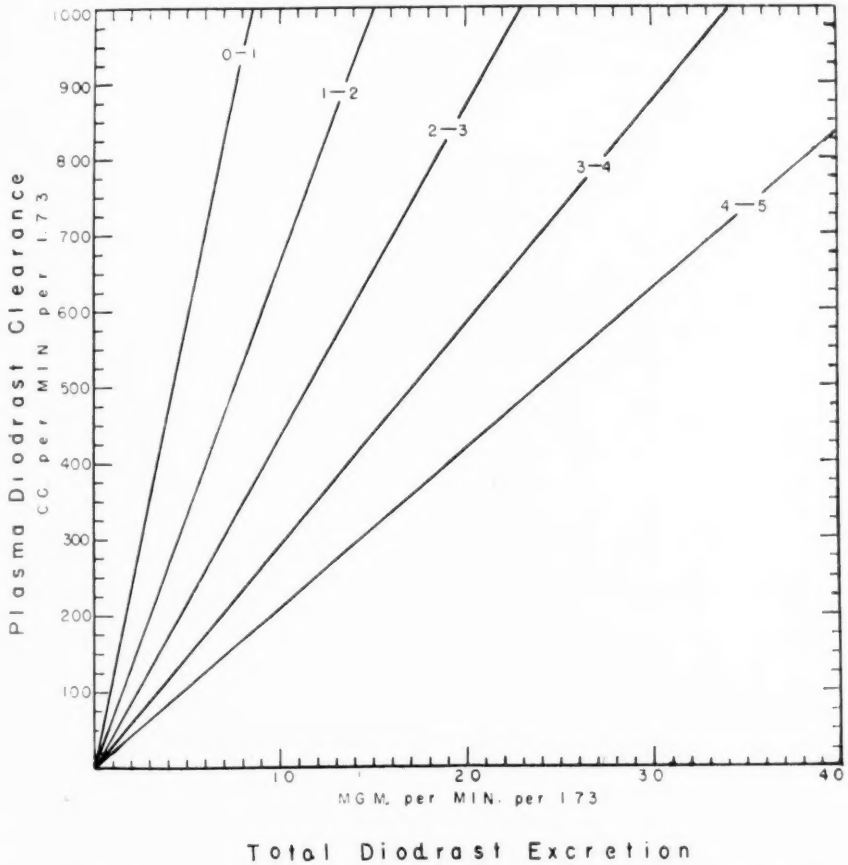

Chart 2.—Correction factors for diodrast-Tm prediction. The numbers on the slanting lines represent plasma diodrast-iodine concentrations in mg. per 100 c.c. From these values and the observed plasma clearances the quantity of diodrast coming from subcutaneous sources during the thirty-minute test may be predicted.

represented by a slanting line and contains plasma clearances plotted against total diodrast excretion rates. Assuming that the plasma clearance of subcutaneous diodrast is not altered by the intravenous injection, one may subtract the predicted total diodrast excretion rate for every minute elapsing between the last two bladder washings. This interval is, of course, variable, since it includes not only the five-minute injection period and the subsequent half-hour collection period, but also such time as may be necessary for obtaining the final

blood sample, changing syringes, etc. It has seemed better to make this correction than to attempt to wash the bladder twice in rapid succession. It is believed that these corrections for subcutaneous diodrast reduce possible errors in Tm estimation not dependent upon technical factors to considerably less than 5 per cent. These correction factors are probably a little too large, since renal diodrast extraction is more nearly complete at low than at high plasma concentrations.

CONCLUSION

A line chart is presented which permits prediction of diodrast-Tm from a single urine specimen. When this is combined with the subcutaneous method for determining renal plasma flow and glomerular filtration rate, the entire procedure is greatly simplified.

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MEDICAL ILLUSTRATION

COLOR PHOTOGRAPHS SIMPLIFIED*

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IT IS generally conceded that color photography is a valuable asset in the presentation of post-mortem material. We have evolved a setup which, we believe, reduces the problems of equipment, expense, personnel, and time to a minimum.

The only equipment required is a miniature camera and a homemade frame of ordinary wood. The color film used is the least expensive made and it costs but ten cents for each picture. The photographic work can be done by a member of the regular staff, thus eliminating the need of specially trained individuals. This is essential since most hospitals do not have sufficient photographic work to employ a full-time photographer. Once the setup is calibrated, it takes less than one minute to take a picture.

The apparatus is shown in Fig. 1. For the most part it is made of wood. The base should be of heavy material to secure immobility and minimize vibration. The overall dimensions are about thirty inches by thirty inches. The area upon which the specimens are placed to be photographed (the millimeter rule in Fig. 1) is eighteen inches square. It is so fashioned that it can be slid up or down upon wedges made for that purpose. The wedges are placed in steps about one and one-half inches apart. The camera is attached by a wooden extension and placed so that the lens is centered exactly in the middle of the plate upon which the specimens are to be placed. The camera must be fixed rigidly and left in the proper position. Two No. 2 photofloods in reflectors (cardboard reflectors would do) are attached to upright posts at either side. The lights are set equidistant from the center of the specimen plate to illuminate evenly the area to be photographed. When satisfactorily arranged they should not be disturbed.

In our particular setup a Kodak Bantam with an Ektar F:2 lens of 45 mm. focal length is used. Almost any miniature camera could be adapted to the purpose. A portrait lens placed in front of the regular lens serves the dual purpose of decreasing the working distance and more fully utilizing the film area. According to the portrait lens used, the size of the image on the film may be increased one to two diameters. This is advantageous because it results in a larger screen image when projected. A polarizing screen may be added to the system to reduce reflection and high lights. Once it has been set to polarize the permanently placed light sources, care should be taken that it is not moved from that position.

*From the U. S. Marine Hospital.
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To calibrate the setup the back of the camera is opened and a piece of ground glass placed at the film plane. The maximum and minimum distances of focus can be determined by placing a millimeter ruler on the specimen board and raising and lowering the board by steps while observing the image on the ground glass. The highest and lowest satisfactory steps are marked and when a small specimen is photographed the highest step is used; the lowest step is used for the large specimens. The board is then placed halfway between these points and with the lens wide open a critically sharp focus is obtained of the millimeter divisions on the ground glass. When this setting has been chosen it should not be moved by jarring. The shutter is then closed and color film inserted. The shutter speed should be set at the longest available time, one second if possible.

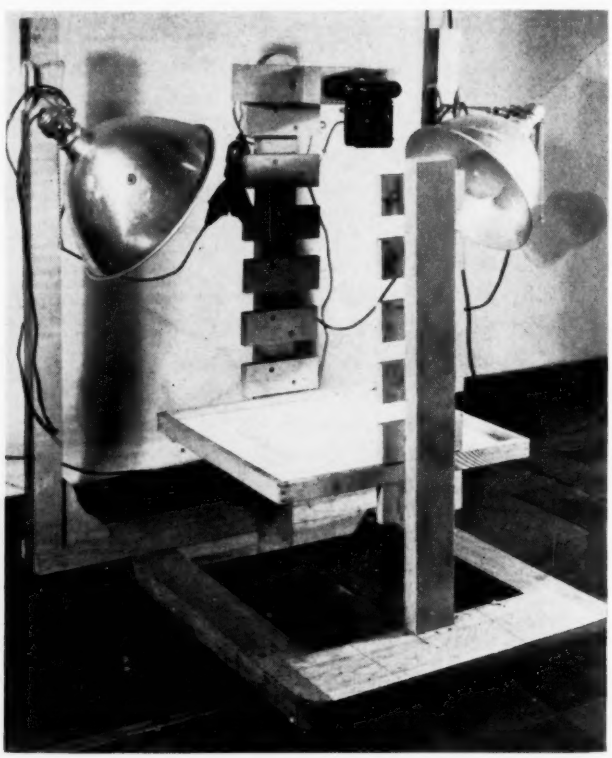

Fig. 1.—Photograph of apparatus showing fixed camera and lights and movable specimen board.

An average specimen, such as a heart, should be used for the calibration. The lens opening is closed to the lowest stop and an exposure made. The frame is moved along, the lens opening increased one stop, and another exposure taken. This procedure is repeated until all available lens openings have been used with the same shutter speed. The purpose of the slowest shutter speed is that the smallest lens opening will be utilized and consequently the greatest depth of focus will be obtained. After the films have been processed they may be projected to ascertain the best exposure. The lens stop is returned to this setting and kept there. The time setting remains stationary. To take subsequent pictures it is

merely necessary to place the specimen in the proper place and make the exposure. The only variable is the specimen.

The method is not without limitations. Exceptionally small and unusually large specimens cannot be reproduced well. Extremes in the color of some specimens, such as a dark blue lung or a white aortic vessel, will not be perfectly exposed at the medial exposure selected.

SUMMARY

A simplified method of taking color photographs of post-mortem specimens is presented. The necessity of taking light readings, focusing the specimen, and making the usual camera and light adjustments is eliminated. By reducing the variables to one (the specimen), excellent results are generally obtained.

BOOK NOTICES

Psychogenic Factors in Bronchial Asthma*

PSYCHOGENIC Factors in Bronchial Asthma consists of two volumes, the first a symposium by several members of The Institute for Psychoanalysis, Chicago, and the second a series of case reviews. Allergists, especially in the early days of this branch of medicine, were guilty of a one-sided outlook in their efforts to explain and cure all asthma entirely on the basis of skin tests. More recently their viewpoint has broadened so that good allergists today give as much attention to nonallergic factors such as focal infections and psychogenic problems, as they do to specific therapy. The reviewer's impression of the monograph under discussion is that the psychoanalyst, having discovered a psychogenic factor in some cases of asthma, has become guilty of the same hyperenthusiasm for his particular approach. The cases studied by the method of dream analysis are too few to be inclusively convincing. The observations are interesting and should be especially so to those allergists who may have devoted too little attention to psychogenic factors. One still wonders what happens to the asthmatic who doesn't dream.

Manual of Clinical Laboratory Methods†

A COMPREHENSIVE, technical, loose-leaf compendium of methods of interest to the technician.

Synopsis of Blood Diseases‡

THE purpose of this small book is to present in succinct form a synopsis of the salient features of diseases of the blood, addressed primarily to the senior student and the practitioner.

This is admittedly a difficult objective. The book is, hence, somewhat difficult to evaluate. It was once said that if the Tallqvist hemoglobin scale served no other purpose than to acquaint the observer with the appearance of drawn blood, a useful purpose was achieved. In like manner, if this book serves only to call attention to the complexities of diseases of the hematopoietic system, it may also serve to emphasize the difficulties embraced in their thorough understanding.

The Rat in Laboratory Investigation§

THIS book represents a compilation of some thirty different collaborators, all well-known workers in their respective fields. It covers everything about the rat, from love at first sight to psychoses and intestinal parasites in the oncoming generation. Every virtue, every weakness, and every pecularity of the rat is fully and completely described. The anatomy,

*Psychosomatic Medicine Monograph IV. Psychogenic Factors in Bronchial Asthma. Part I. By Thomas M. French, M.D., and Franz Alexander, M.D. With the Collaboration of various contributors. Paper, 92 pages. Volume II, Nos. I and II. Paper, 236 pages. Published with the Sponsorship of the Committee on Problems of Neurotic Behavior, Division of Anthropology and Psychology, National Research Council, Washington, D. C., 1941.

†Manual of Clinical Laboratory Methods. Opal E. Heppler, Ph.D., M.D., Assistant Professor of Pathology, Northwestern University Medical School. Planographed, 159 pages. John S. Swift Co., Chicago.

‡Synopsis of Blood Diseases. A. Piney, M.D., M.R.C.P. Physician, St. Mary's Hospital for Women and Children, London; Director, Pathological Department, Royal Cancer Hospital, London. Cloth, 120 pages, 4 colored plates, $2.75. The Blakiston Company, Philadelphia, Pa.

§Edited by John Q. Griffith, Jr., M.D., Associate in Medicine, School of Medicine, University of Pennsylvania, and Edmond J. Farris, Ph.D., Executive Director and Associate in Anatomy, Wistar Institute of Anatomy and Biology. Cloth, 488 pages, 178 illustrations, $7.50. J. B. Lippincott Company, Philadelphia, Pa.

physiology, biochemistry, pathology, psychology, and the full usefulness (or the lack thereof) of the rat in laboratory or experimental work is described in minute detail. Special chapters are devoted to breeding, diet, the teeth, digestion, metabolism, the central nervous system, psychologic phenomena, the circulatory system, biologic assay, hematology, osteology, radiology, histology, parasites and special diseases of the rat. Of particular interest are special sections on the eye of the albino rat, the surgery of the rat, and, of especial usefulness, a very extensive chapter (by Dr. Harold G. O. Holck) on dosage of drugs for rats. This latter chapter lists therapeutic and fatal dosages (when given in various ways) for some five hundred different drugs and is followed by a bibliography of 447 references. The chapter on "Technics for the Investigation of Psychological Phenomena in the Rat" (by Dr. George L. Kreezer) is followed by 678 references.

Well printed and admirably illustrated on excellent paper, this book is destined to become a sort of universal encyclopedia and reference work for all those who breed or use rats in experimental or investigational fields.

Macleod's Physiology in Modern Medicine*

SINCE the death of Dr. MacLeod his textbook on physiology has been edited by Philip Bard, Professor of Physiology at Johns Hopkins University School of Medicine with the collaboration of nine outstanding physiologists and pharmacologists. This is the ninth edition, the second under the new editorship. The usual number of additions and alterations have been incorporated, necessitated by advancing knowledge in the field. The work is so widely known that it does not require elaborate review. The paper used is especially desirable. Having a very faint green tinge, it is more restful on the eyes than the more usual white paper. The bibliography appears at the back of the book. It contains an innovation in that at the top of each page there is an index file referring to the pages in the book where the references appear.

Diseases of Metabolism†

DUNCAN'S *Diseases of Metabolism* is a contribution by fifteen outstanding contributors, well qualified in their fields, edited by Duncan, who has also contributed several sections. The subject matter adheres strictly to the title. The first half of the book deals with the metabolism and physiology of carbohydrates, fats, proteins, minerals, water, and vitamins. Expressed this way, one might have the impression that the discussion is a rehash of information long since available. Quite on the contrary, it consists of a splendidly arranged review of the abundant recent developments in the field. The first half of the volume alone places it in the category of better than average medical texts. It correlates much that has heretofore been available mostly in scattered articles.

Metabolic disturbances discussed in detail include undernutrition, obesity, xanthomatoses, gout, hyperinsulinism, diabetes insipidus, melituria and diabetes mellitus. The only chapter which might well have received more extensive discussion is that on undernutrition.

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*Macleod's Physiology in Modern Medicine. Edited by Philip Bard, Professor of Physiology, Johns Hopkins University School of Medicine. With the Collaboration of Henry C. Bazett, Professor of Physiology, University of Pennsylvania, George R. Cowgill, Associate Professor of Physiological Chemistry, Yale University School of Medicine; Howard J. Curtis, Instructor in Physiology, Johns Hopkins University School of Medicine; Harry Eagle, Passed Assistant Surgeon, United States Public Health Service and Lecturer in Medicine, Johns Hopkins University School of Medicine; Chalmers L. Gemmill, Associate in Physiology, Johns Hopkins University School of Medicine; Magnus I. Gregersen, Professor of Physiology, College of Physicians and Surgeons, Columbia University; Roy G. Hoskins, Director of Research, Memorial Foundation for Neuro-endocrine Research; Research Associate in Physiology, Harvard Medical School; J. M. D. Olmsted, Professor of Physiology, University of California; Carl F. Schmidt, Professor of Pharmacology, University of Pennsylvania. Ninth Edition. Cloth, 1256 pages. The C. V. Mosby Company, St. Louis, 1941.

†Diseases of Metabolism. Detailed Methods of Diagnosis and Treatment. A Text for the Practitioner. Edited by Garfield G. Duncan, M.D., Chief of Medical Service "B." Pennsylvania Hospital; Associate Professor of Medicine, Jefferson Medical College, Philadelphia, Pennsylvania. Contributors: Walter Bauer, Abraham Cantarow, Garfield George Duncan, Ferdinand Fetter, Cyril Normal Hugh Long, Louis Harry Newburgh, Tom D. Spies, Hugh R. Butt, Tracy Donald Cuttle, Frank Alexander Evans, Friedrich Klemperer, Edward Halton Mason, John Punnett Peters, Leandro Maués Tocantns. Abraham White. Fully illustrated including 7 plates in color. Cloth, 985 pages. W. B. Saunders Company, Philadelphia and London, 1942.

The Avitaminoses*

NEW facts are evolving so rapidly in the study of vitamins that it was fortunate that Eddy and Dalldorf's *Avitaminoses* required a second edition after three years. Even so, the authors state that advances in our understanding of the vitamins necessitated a complete rewriting of most of the text. This volume will be of service to biochemists, clinicians and nutritionists alike. The first portion deals with the chemical nature of the vitamins. There follows a chapter on the functions of the vitamins in cellular oxidation which will enable the average reader to comprehend more clearly the action of these food components. The major portion of the volume is given to the description of the nature and function of each vitamin, the physiologic changes accompanying deficiency and the results of treatment. In an appendix, laboratory tests for vitamins are described and there is an excellent table of the vitamin content of most foods.

A Study of the Blood in Cancer†

IF ONE would read the last page of *The Blood in Cancer* first, one would be inclined not to read more. The observations are reminiscent of "Doctor" Abrams and Huckleberry Finn's reaction to the light of the moon.

Going backward in this volume, part III, describes many tests for cancer which have been recommended by various authors including urine tests, enzyme tests, and the like.

The major portion of the book deals with the author's studies of blood cytology in cancer. Dr. Gruner believes that unusually viscid blood without rouleaux but with tight cohesion of the red cells into islands with sharply defined contours and an early appearance of a dense fibrin network with granular matter in the meshes and actively moving leucocytes, reduction in the lymphocytes, irregular nuclear contours of the neutrophils, large polymorphous platelets, abnormality in the contour of the nucleus in the monocytes, and mitochondrial inclusions in the mononuclear leucocytes usually indicates malignancy.

SIMPSON MEMORIAL INSTITUTE
ANN ARBOR, MICH.

*The Avitaminoses. The Chemical, Clinical and Pathological Aspects of the Vitamin Deficiency Diseases. By Walter H. Eddy, Ph.D., Professor of Physiological Chemistry, Teachers College, Columbia University; Director, Bureau of Foods and Sanitation, "Good Housekeeping Magazine" and Gilbert Dalldorf, M.D., Pathologist to the Grasslands and Northern Westchester Hospitals, Westchester County, N. Y. Second Edition. Cloth, 519 pages, $4.50. Williams & Wilkins Company, Baltimore, 1941.

†A Study of the Blood in Cancer with Special Reference to the Needs of the Tumour Clinic. Fully illustrated, including graphs and coloured drawings. By O. Cameron Gruner, M.D. (Lond.). Author of "Biology of the Blood Cells" and "Studies in Puncture-Fluids." Cloth, 100 pages, $4.00. Renouf Publishing Company, Montreal, 1942.